The assessment of reticulocyte and erythrocyte haemoglobin contents, and their use in the evaluation of iron status in hospitalised patients

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Abstract

Current clinical practice relies on sufficiently low haemoglobin (HB) level, low serum ferritin (SF), low serum iron concentration and an elevated total iron binding capacity (TIBC) to identify iron deficiency (ID) and iron deficiency anaemia (IDA). However, these parameters are sometimes not reliable in assessing iron status in patients with underlying conditions like chronic diseases, malignancies and inflammation. Growing evidence indicates the appropriate inclusion of reticulocyte haemoglobin content (RET-He) and red cell haemoglobin content (RBC-He) into the iron studies investigation could improve the early diagnosis. The aim of this study were to evaluate the extent to which RET-He and RBC-He values could improve the assessment of iron status in chronic disease and inflammation, and to evaluate if the inclusion could be used to preclude the need for further biochemical assessment in iron status.

A total of 800 samples (89 normal, 611 patients and 100 haemochromatosis) were obtained and analysed for biochemical and haematological iron indicators. RET-He and RBC-He were added to the full blood counts parameters on the Sysmex XE2100^{TM}, and C-reactive protein was added to indicate inflammation. The inclusion criteria were based on symptomatic and asymptomatic cases, such as gastroenterological, cancer and rheumatoid arthritis. Children and pregnant women were excluded from participating in the study. Stability of RET-He, RBC-He, HB, mean cell volume (MCV) and mean cell haemoglobin (MCH) were evaluated to determine if there was significant shift in the result over 96 h and at varying temperature 4^0C, 20^0C and 30^0C. RET-He and RBC-He were also added to the haematological iron assessment in the follow up monitoring of haemochromatosis patients to evaluate their usefulness.

The serial measurement shows reasonable stability for MCH, HB, RET-He and RBC-He for up to 96 h. Elevated shift were recorded for MCV on all the samples in the same time frame. Receiver operating characteristic (ROC) analysis was used to obtain sensitivity and specificity of the biochemical and haematological iron indices measured in the study. Female patients were classified into ID using SF ≤ 20 ng/mL. The area under the curve (AUC) in this group were (SF = 0.99 versus RET-He and RBC-He = 0.72). RET-He has (sensitivity, 89.6% and specificity, 58.0%), and RBC-He, (sensitivity 88.6% and specificity 57.0%) in detecting ID. Female patients with IDA were classified using HB ≤ 11.5 g/dL. The AUC were (HB = 0.99; versus RET-He = 0.87 and RBC-He = 0.86). RET-He has (sensitivity, 84.0% and specificity, 72.0%) and RBC-He (sensitivity 82.0% and specificity 71.0%) versus HB (sensitivity, 100.0% and specificity, 99.9%) in detecting IDA. In male patients with ID, SF < 20 ng/mL was used for classification, AUC were (SF = 1.00 versus RET-He = 0.78 and RBC-He = 0.79). RET-He has (sensitivity, 78.4% and specificity, 66.0%) and RBC-He (sensitivity 80.0% and specificity 61.2%), against SF (sensitivity, 100.0% and specificity, 100.0%) in detecting ID. In male patients with IDA, HB <13.5 g/dL was used for classification, AUC were (HB = 0.99 versus RET-He = 0.89 and RBC-He = 0.87). RET-He has (sensitivity, 88.6% and specificity, 83.0%) and RBC-He (sensitivity 83.8% and specificity 80.0%) in detecting IDA in male patients. Diagnostic plots were used to assess the ability of RET-He and RBC-He in comparison to other biochemical and haematological parameters, RET-He and RBC-He emerged as possible predictors of ID and IDA in inflammations and chronic diseases. The results of RET-He and RBC-He when measured with the other haematological parameters in the follow up treatment of haemochromatosis patients, shows 99% agreement with HB, MCV and MCH.

In conclusion, the inclusion of RET-He and RBC-He into the iron studies investigation may improve the identification of ID and IDA in chronic

disease and inflammation, and can possibly detect changes in the cohort of cells with inadequate haemoglobinisation. Therefore, both indices hold promise as an alternative to biochemical iron studies especially, in patients with acute phase response and chronic diseases.

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Abbreviations

ACD	Anaemia of chronic disease
CRP	C-reactive protein
CV	Coefficient of variation
ELISA	Enzyme linked immunosorbent assay
FERR	Serum ferritin
FID	Functional iron deficiency
fL	Femtolitre
HB	Haemoglobin
ID	Iron deficiency
IDA	Iron deficiency anaemia
MCH	Mean cell Haemoglobin
MCHC	Mean cell haemoglobin concentration
MCV	Mean cell volume
pg	Picogram
RBC-He	Red blood cell haemoglobin content
RDW	Red cell width distribution
RET-He	Reticulocyte haemoglobin content
SD	Standard deviation
sTfR	Serum transferrin receptor
TIBC	Total iron binding capacity
TSAT	Transferrin saturation
UIBC	Unsaturated iron binding capacity
WHO	World Health Organisation
ZPP	Zinc protoporphyrin

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Dissemination

Posters and presentations

The author submitted part of the work documented within this thesis as a poster to the Academy of Medical Laboratory Science in Ireland. The poster entitled: An assessment of red cell haemoglobin equivalents (RET-He and RBC-He) in screening for iron deficiency, was accepted and presented at the 2007 national conference in Sligo, Ireland.

Declaration

I declare that whilst registered as a candidate for the above degree, I have not been registered on any other research award. The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other academic award.

The work contained within this submission is my own work, and to the best of my knowledge and belief, it contains no material previously published or written by another person, except where due acknowledgement has been made in the text.

Aderemi O. Adelugba

August 2012

Dedication

I wish to dedicate this thesis to God almighty, who has given me the strength and life to be able to pursue this work to completion.

To my wife, Aramide and my children David, Samuel and Hannah for your constant support and encouragement throughout this research work. I know you are all proud of me to have reached this point.

CHAPTER 1 Literature review

1.0 Introduction

Iron status is described as a continuum from iron deficiency with anaemia or iron deficiency without anaemia to normal iron status with varying levels of storage iron, and finally to iron overload which results in haemochromatosis which can consequently lead to severe organ damage if untreated (World Health Organisation, 2001, p.3).

The World Health Organisation (WHO) is one of many organisations that has highlighted the seriousness of iron deficiency and anaemia caused as a result of its depletion. Iron deficiency (ID) is probably the most frequent nutritional deficiency in the world (Bohmer *et al*., 2003). More than two billion people, equivalent to over 30% of the world population suffer from the most serious type of ID with significant and obvious signs of iron deficiency anaemia (IDA) (Bohmer *et al*., 2003). ID was identified as one of the ten leading risk factors for disease, disability and death in the world, with a much greater impact than either vitamin A or zinc deficiency. The prevalence of IDA is believed to be around 2-5% amongst men and post-menopausal women in the developed world (World Health Organisation, 2001, p.3).

Iron plays an important role in health and disease (Conrad, 1999). In fact Bryan (1931) indicated that documented therapeutic uses of iron dated back

as far as 1500 BC. However, it was not until 1872 that iron was recognised as a vital nutrient for animals (Boussingault, 1872). Iron is an essential trace element and the key component of many cellular enzymes, such as catalases, peroxidases, cytochromes, ribonucleic reductases, aconitases, and nitric oxide synthases, all of which are required for metabolic and oxidative processes intrinsic to life (Boldt, 1999; Conrad, 1999). These enzymes can be implicated in the development of a wide range of diseases, such as iron disorders, cancers, neurodegenerative diseases, and ageing (Conrad, 1999). Iron represents approximately 35 mg/kg and 45 mg/kg of body weight in adult women and men respectively (Bothwell *et al*., 1995). The average western diet contains 10-15 mg of iron, from which only 5-10% is absorbed (Bothwell *et al*., 1995).

Before the twentieth century, most advances in the biochemical assessment of iron seemed to parallel, rather than complement the developments seen in haematology. Central to all of these are the processes involved in the absorption of iron, which detect and respond to the requirement in the body. The process is known as the physiology of iron.

1.1 Physiology and functions of iron

Iron incorporated in haemoglobin in red blood cell is required for the transport of oxygen by the blood to all organs in the body. It is essential for

growth, brain cell development, physical activity and capacity to work (Beard, 2001). Iron is transported by haemoglobin (HB) in the blood, and when HB is rich in oxygen, it is red. When ID is present, there is less HB (World Health Organisation, 2004). Iron is involved in electron transport and has physiological functions which guide the availability of iron in the body. Therefore, too little iron can interfere with these vital functions and possible morbidity and mortality may be the outcome (Centre for Disease Control, 1998).

An adequate supply of iron is required for normal haemopoiesis (blood cell formation), which is important in the immune response system and helps white blood cells produce compounds necessary in fighting bacteria (Fairbank & Klee, 1999; Beard, 2001; World Health Organisation, 2004).

In normal adults, 0.5-2 mg of iron is lost daily due to blood loss and the constant exfoliation of iron-containing epithelial cells that line the gastrointestinal and urinary tracts, skin and hair. To maintain homeostasis, the same amount of iron is required each day to replace the lost iron (Sharp *et al*., 2007). Excessive iron can lead to the formation of reactive radicals and can cause cell damage. This can also contribute to the development of numerous iron disorders, neurodegenerative diseases and possibly cancers (Sussman, 1992). In human cells, the expression of many key molecules that are involved in iron metabolism is controlled by the level of intracellular iron

via feedback mechanisms through gene expression at the post transcriptional level and specific mRNA-protein interactions in the cytoplasm (Haile, 1999).

1.2 Dietary sources of iron

Dietary iron exists in two forms, haem (found almost exclusively in meat) and non-haem (found in plants and animals). The main contributors to iron intake in the United Kingdom are fortified cereals, bread, meat products and vegetables. The average diet contains about 10-15 mg of iron daily. This is made available in the form of haem proteins, haemoglobin and myoglobin, the majority of which are derived from meat (Roach & Benyon, 2003). Previously, inorganic iron in the diet came from iron cooking utensils. In historic times, Justus von Lieberg, a Professor of Organic Chemistry at the University of Giessen, who carried out various experiments on animal physico-chemical systems, advocated the science of biochemistry. His view was that the minerals in the body came pre-formed as organic compounds derived from plant foods, and this view was widely accepted (Lieberg, 1842). Many other scientists in that era also propagated the views of Lieberg (Curtis, 1906; Chittenden, 1907). However, Gustav von Bunge, Professor of Physiology at the University of Basle, who followed in Lieberg's footsteps, heralded uncertainty over Lieberg's view, he however, suggested that 'the habitual consumption of foods poor in iron may lead to anaemia. He also said

that, ‘it is difficult to imagine a diet that would not contain the small amounts of the metal required daily’ (Bunge, 1902). Bunge later showed in further studies that some foods were low in iron, particularly human milk, and recognised the high level of iron in spinach, egg yolks, beef, apples and lentils which are required for consumption on a regular basis to prevent an individual from suffering from anaemia (Bunge, 1902). More surprisingly, he indicated that iron from haemoglobin in meat was poorly absorbed, which is in contrary to recent evidence that haem is rapidly absorbed from the gastrointestinal tract (Roach & Benyon, 2003).

1.3 Absorption of iron

The intestine serves as the major control for iron regulation. It controls the uptake of dietary iron across the membrane to the circulation. In the lumen, iron exists as ferrous and ferric iron salts. This happens because ferric iron becomes insoluble at pH ≥ 3 and ferric iron must be reduced by amino acids or sugars to be efficiently absorbed and most ferrous iron is soluble at pH 7 (Conrad *et al*., 1999). Therefore, absorption of the ferrous iron salt is more efficiently processed than the ferric iron salts (Conrad *et al*., 1999). As most dietary iron exists in the form of ferric iron salt, its reduction becomes necessary for efficient absorption, and this can be achieved by the interaction of the intestinal enzyme mucosal ferrireductase (Ekmekcioglu *et al*., 1996).

Alternatively, the absorption of ferric iron may be processed in a less effective way by the mediation of the paraferritin pathway (Han *et al*., 1995). Any form of reduction in the activities of these enzymes will result in reduction of iron absorption, which may consequently lead to an iron disorder.

The nutritional absorption of both haem and non-haem iron uptake occurs in the proximal small intestine, specifically in the duodenum and jejunum (Wood & Han, 1998). Enterocytes located on the intestinal villi are highly specialised and polarised absorptive cells which control the passage of dietary iron in the lumen and transfer into the body's circulation (Wood & Han, 1998). The process undertaken before its transfer into the body's circulation involves three cellular membrane barriers: iron absorption across the apical membrane, intracellular membrane and then into the circulation. This transfer process is facilitated by a divalent cation transporter known as "natural resistance-associated macrophage protein", and it helps transport iron from the duodenum lumen into the cytoplasm of epithelial cells (Gruenheid *et al*., 1995). Haemoglobin iron from food is absorbed more efficiently from inorganic iron, the absorption of iron from myoglobin and haemoglobin differs distinctly from inorganic iron (Conrad *et al*., 1999). The first stage involved in the haem iron uptake involves the enzymatic digestion of haemoglobin iron in the lumen. The haem molecules then proceed into the absorptive cell as an intact metalloporphyrin. Following this, haem molecules

enter the cells via the haem mediated receptor-mediated internalisation process (Mills & Payne, 1995). Haem is degraded in the enterocytes by haem oxygenase to yield inorganic iron. Inorganic iron is either stored as ferritin or transported into the body's circulation. Once this process has been completed by the enterocytes, iron that remains in the form of ferritin will be degraded with the senescent cells and excreted through the gastrointestinal tract. This process represents an important mechanism of iron loss (Mills & Payne, 1995).

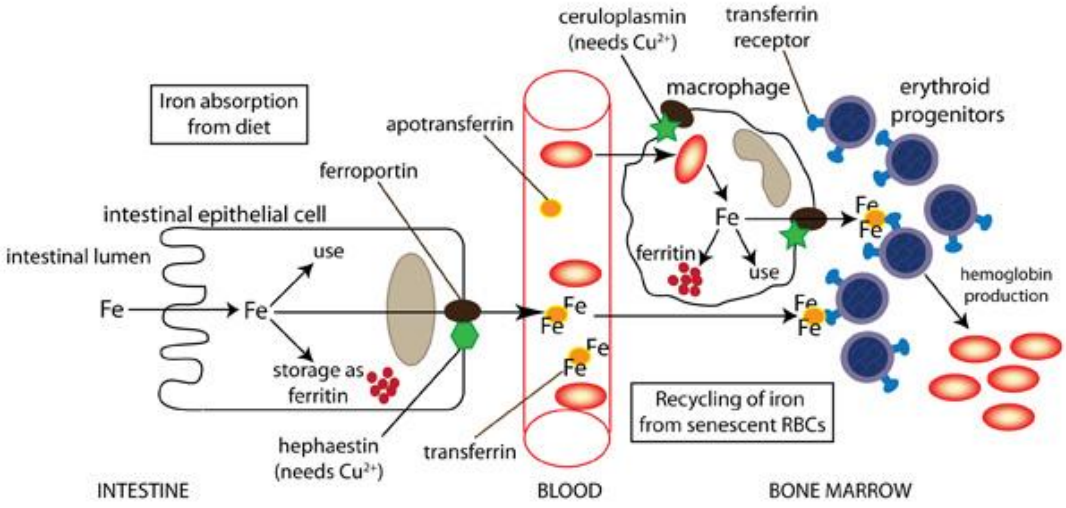


Figure 1.1: Iron absorption, storage and transport pathway (Reproduced with permission from Andrews, N.C. (1999, pp. 1986-1995).

Iron absorption by the enterocytes in the gastro duodenal junction is regulated in several ways (Figure 1.1). It can be modulated by the amount of

iron consumed in the diet, known as “dietary regulator” (Andrews, 1999). Also, following adequate consumption of iron in the diet and depending on the cellular absorptive ability, the enterocytes may resist further acquisition of iron, this process is known as “mucosal block” (Andrews, 1999). The second regulatory mechanism will sense the body stored iron levels rather than the dietary iron status, this mechanism is termed the “store regulator”. The store regulator can influence the uptake of iron by about two to three factors in iron-deficient conditions, and the final mechanism is known as “erythropoietic regulator”. This has a greater capacity to increase iron uptake in response to the requirement for erythropoiesis (Andrews, 1999).

1.4 Iron metabolism

An important issue of iron metabolism in man is that it is very conservative, because there is no controlled excretory mechanism. Iron is recycled to the extent that the movement of iron within its compartment is described as an iron cycle.

Iron homeostasis is maintained by regulating its import, that is, during times of deficiency its uptake is stimulated, whilst under iron-rich conditions, uptake is suppressed to prevent toxicity (Smith *et al*., 1992).

The majority of total body iron (about 60-70%) is found in the haem compounds, especially in the form of haemoglobin and myoglobin, while the storage forms of iron, ferritin and haemosiderin, consist of about 30%, and only a minute amount (0.2%) is carried and bonded with a transport protein, transferrin (Brugnara, 2000). The production of two-three million red blood cells every second requires an adequate supply of iron, and iron moves freely from plasma to the marrow cells that have the capacity to make haemoglobin, this process allows the developing red cells to synthesise sufficient haemoglobin, so that they emerge into the bloodstream with intracellular haemoglobin as mature red cells (Conrad *et al*., 1999). This is a dynamic process that enables a total daily output of 6 g of haemoglobin from the marrow, as it is required for the production of erythroblasts (Brugnara, 2000). Iron metabolism takes place in specialised tissues; the testes, intestines, placental and skeletal muscles, and high levels of iron are found in the liver, erythrocytes, brain and macrophages (Andrew *et al*., 1999).

Iron uptake by the body is regulated by a peptide hormone known as hepcidin. When body stores of iron are high, the liver secretes hepcidin (Park *et al*., 2001). Hepcidin has been identified as the key regulator in systemic iron mobilisation. It was first identified in urine as a small bactericidal peptide (Krause *et al*., 2000; Park *et al*., 2001). Hepcidin is a 25-amino-acid molecule which is closely similar to the defensin antimicrobial peptides (Krause *et al*, 2000; Park *et al*, 2001). It explains how iron release from absorptive

enterocytes and macrophages is controlled within the circuit. The first connection between hepcidin and iron metabolism was ascertained by Pidgeon *et al*., (2001). Hepcidin is noted to decrease iron absorption in the intestine and also block the export of ferroportin, which is responsible for iron efflux into the cells when required for erythropoiesis (Nemeth *et al*., 2004).

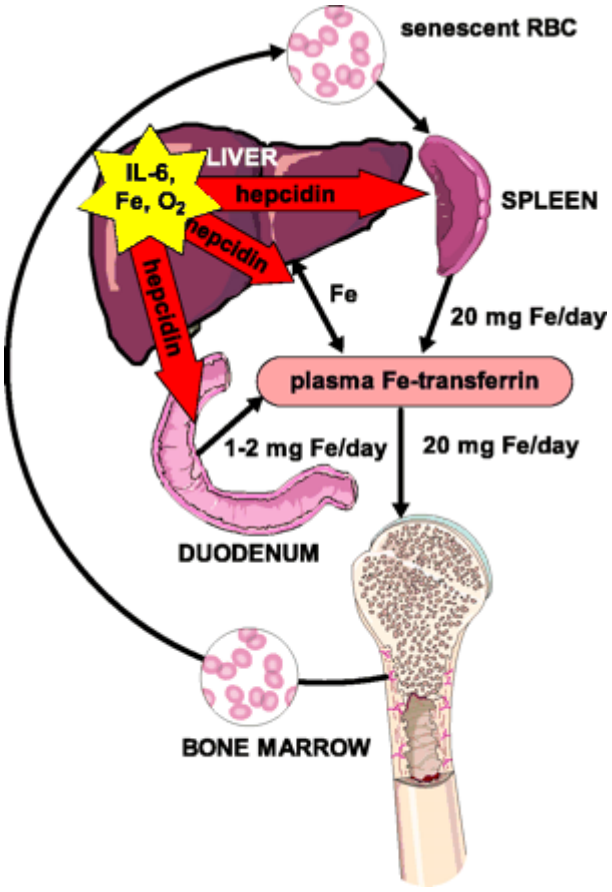


Figure 1.2: Role of hepcidin in iron regulation (Reproduced by permission from Ganz & Nemeth, (2006, pp. G200).

Ferroportin is a transmembrane protein that serves as the sole cellular efflux channel for iron from the inside of a cell to the outside of it. It is found on the surface of the cells that transport and store iron including enterocytes, hepatocytes and macrophages. The efflux is regulated by the iron regulatory hormone hepcidin. The process involves internalisation and degradation of ferroportin, which is available on the cell surface of macrophages and enterocytes, and causes iron to become trapped within enterocytes, hepatocytes and macrophages leading to reduction in iron uptake. It has been identified during inflammation that the cytokine interleukin-6 alone can rapidly induce hepcidin production and result in corresponding hypoferraemia (Nemeth *et al*., 2004). The process of iron metabolism shows that excess intracellular iron can possibly lead to the formation and deposition of haemosiderin which can lead to cellular dysfunction and damage, also the consequence of excess iron intake is haemochromatosis (Gerlach *et al*., 1994).

The biological importance of iron is in its chemistry, because it exists in both ferrous (Fe^{2+}) and ferric (Fe^{3+}) oxidation states, which means iron is capable of accepting and donating electrons, this oxidation-reduction reaction is known as Fenton reaction (Weissling-Ressnick, 1999).

$Fe^{3+} + O_2^{-} \rightarrow Fe^{2+} + O_{2;}$ $\quad$ $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH + OH^{-}$ $\quad$ (Eqn 1)

The reaction above (Eqn 1) is important for the biochemical functioning of iron especially in cellular metabolic processes. The deleterious effect of iron can be likened to its ability to produce reactive oxygen species within the Fenton reaction (McCord, 1998). The highly reactive oxygen species like the hydroxyl (OH^-) and superoxide radicals (O_2^-) are very toxic because of their ability to react with every molecule found in the living cells. This can result in DNA damage, impaired synthesis of proteins, membrane lipids, and sometimes altered cell proliferation (Smith *et al*., 1992; McCord, 1998).

The process described above has led to suggestions that iron plays a role in carcinogenesis, the pathogenesis of atherosclerosis and neurodegenerative disorders such as Parkinson's or Alzheimer's disease (Jenner, 1991; Connor *et al*., 1992). Therefore, to reduce the effect of this toxicity using a highly sophisticated mechanism, molecules for the acquisition and transport of iron in soluble, non-toxic forms have evolved to meet cellular iron needs, and also to control homeostasis. Although the amount of iron extracted from the diet is small, regulation of intestinal absorption is critical because humans have no active physiological pathways for excretion. Duodenal crypt cells sense the iron need of the body by taking up iron from plasma transferrin at the basolateral surfaces and migration to the villus region. Therefore, iron levels are thought to be predetermined in the crypt cells and control the expression of iron transporters in the body as they mature into absorptive enterocytes lining the absorptive villi close to the gastrointestinal junction (Andrews *et*

al., 1999). Physiological iron loss from the body includes minute excretion from the bile, urine and daily loss through the gut and skin, which represent about 1 mg/day in adults (Andrews *et al*., 1999). Women experience additional iron loss through monthly menstrual bleeding and during pregnancy. The balance of red cell production and destruction maintains the HB level. When production fails to keep up with the destruction, either as a result of blood loss through disease or injury, or reduced dietary intake of iron, there will be a reduction in the HB level until it reaches an arbitrary level to indicate anaemia (negative iron balance).

1.5 Factors influencing iron bioavailability

There are numerous factors which affect iron absorption and bioavailability. The factors are age, iron status, species, dosage level and organic and inorganic nutrients. McCance & Widdowson (1937) proposed that efficiency of absorption was due primarily to the iron status of the animal. Bothwell *et al*. (1995) also indicated that the two most important factors influencing iron absorption were iron stores and rate of erythropoiesis. This substantiates the mucosal block theory that a human only absorbs what it needs or requires (Andrews, 1999). Increasing levels of dietary iron lead to higher total amounts absorbed and, because of its potential ability to cause deficiency or overload, iron status remains the most influential in determining its

absorption, and that is why it is very necessary to study iron status and deduce an improved way of assessing its levels in health and disease.

1.6 Victorian era of anaemia

From the 16th to the early 19th centuries, several interesting historic issues regarding the lack of iron in the body have been discussed. Anaemia, hypochromia, and lack of iron in the blood were first discussed in the 1830s by Hoefer, Popp, and Foedisch respectively (Fowler, 1936), and many workers continued to improve the understanding of this discovery. Several conditions were used to describe the clinical effects of ID in the medical literature, followed by the terminology *anaemia* meaning lack of blood or a decrease in red blood cells.

In 1836, Ashwell defined chlorosis as a disease of the blood characterised by anaemia and lack of iron. Chlorosis, also called 'green sickness', morbus virgeneus, mal d'amour, is a fascinating disorder resulting in ID in adolescent girls between the ages of 14 and 17 years with gastrointestinal and menstrual disorders, and its prevalence peaked in the Victorian era (Patek & Heath, 1936; Guggenheim, 1995). The term chlorosis was frequently used and adopted in medieval ages and then completely disappeared as a clinical entity before World War II (Hart, 2001). The hypothesis adopted to explain chlorosis compared the disease with the use of tight lacing of the body with

corsets or other materials worn by girls and women in the 16th century. The prevalence was so high during this period that scientists thought wearing these garments could have possibly compressed the liver, which is believed to have caused displacement of the abdominal organs, even though this must have resulted from other causes like reflux oesophagitis resulting in blood loss, other than mere compression (Hansen, 1931). In today's terms, we do not regard even severely anaemic individuals as green. The nomenclature has changed. Most of the features associated with chlorosis are apparent in IDA. The picture of chlorosis was viewed as a combination of numerous factors relating to demands of growth and the onset of menstruation, inadequate diet and possibly lack of iron stores at birth as a consequence of ID in mothers. Patients who suffered from chlorosis responded very well when treated with iron replacement pills (Haden, 1938).

Another form of ID which has been described after the demise of chlorosis is simple achlorhydric anaemia. Others referred to it as idiopathic or primary hypochromic anaemia (Witts, 1931; Wintrobe & Beebe, 1933). Like chlorosis, this type of anaemia mainly affected women, especially those with poor diets, menstrual problems and multiple pregnancies, and it occurs later in life around the ages of 40-50 years (Wintrobe & Beebe, 1933). Following some studies carried out, the pathogenesis of this condition has become clearer, and it was identical to what is known as IDA nowadays. Therefore, it is called chronic hypochromic anaemia. The clinical features include epithelial changes

seen in nails, tongues and achlorhydria. Patients suffering from chronic hypochromic anaemia were found to be responsive to iron therapy, although there were controversies concerning the dosage and preparation of iron that was required to treat the disease (Haden, 1938).

1.7 Iron deficiency: physiological stages

The physiological disorder of iron metabolism is related to an increased iron need during growth and developmental phases, while the pathological causes are as a result of loss of iron due to a serious or chronic medical condition, which also depends on a range of factors, such as age, co-morbidity, and concomitant therapies (Rockey & Cello, 1993). The overall understanding is that ID results from an imbalance of supply and demand for iron in the body, whereby the requirement versus rate of absorption is not met. Hence, patients with ID possibly have inadequate intake, impaired absorption, suppressed transport or iron losses as a result of reproductive age or chronic blood loss. Despite various established diagnostic protocols and guidelines, many patients suffering from ID still lack definitive aetiology, and this makes it difficult to establish and identify the condition more accurately (Rockey & Cello, 1993). ID is the most frequent cause of anaemia worldwide, affecting a large number of children and women, especially in developing countries (Nojilana, *et al*., 2007). It is also the only nutrient deficiency which is

prevalent in highly industrialised countries with the number currently standing at two billion people, over 30% of the world's population (World Health Organisation, 2010). The fundamental aetiologies are a result of inadequate amounts of iron being consumed, and an excessive blood loss with disorders in its metabolism (Pollitt, 1993). A wide range of physiological states including pregnancy and aging have also been implicated. This negative iron balance resulting in exhaustion of total body iron stores and diminishing SF ≤ 12 µg/L is always indicative of ID with or without anaemia (Goddard *et al*., 2000; Jolobe, 2000). The magnitude of the problem of ID and its effect on global health is well appreciated, especially by the people who have been working in the field of nutritional studies. It is quite obvious that clinical interests have focussed on early diagnosis of ID, to prevent patients from progressing to a more serious and acute disease stage. IDA still remains the most common treatable anaemia in the world, once the cause of the underlying anaemia is identified. Major fundamental progress has been introduced for physiologic and pathologic connections between iron metabolism and erythropoiesis, which include multiple genes and factors which regulate erythropoiesis (Orkin, 2000). ID without overt anaemia can result in neuropsychological effects and has been linked to delayed cognitive development in children and adolescents (Grantham-McGregor & Ani, 2001). This delay has been shown to respond to iron therapy (Sandstead, 2000).

The stage of development of ID is usually sequential. It spans over a long period of negative iron balance. As the body iron begins to fall, there is a characteristic pattern of events that results in three stages in which deficiency of iron is seen. The first stage is known as the iron depletion stage, which is also known as the pre-latent ID stage. For the majority of the time during this stage, even though there is iron depletion, the diagnosis using serum iron cannot be made as serum iron is not necessarily reduced due to the iron supply from storage molecules (Choi, 2003). Therefore, detection of ID at this stage would depend on the measurement of serum ferritin (SF). A low SF concentration is sensitive for appraising iron depletion when there is no inflammation. However, its use has limitations, because it can be elevated by any type of chronic inflammation. The use of biopsy techniques can be employed, but this method is invasive.

The next stage of deficiency is known as latent ID. This is the stage where iron depletion is complete and storage has been exhausted. The HB level is mostly higher than the lower reference limit during this stage and the mean cell volume is usually normal. This stage presents a characteristic difficulty for clinicians to diagnose using haematological parameters alone. Therefore, some biochemical parameters are always requested. On most occasions decreased concentrations of ferritin and transferrin saturation are useful in the diagnosis, and an elevation of erythrocyte protoporphyrin have been utilised in the diagnosis (Heinrich, 1968; Verloop, 1970; Köller, *et al*., 1978).

The final stage in the development of ID is the progression from the state of deficiency of iron to anaemia. This is the stage where the HB concentration falls below the lower limit of the reference interval, and is known as IDA.

1.8 Features of iron deficiency

The generic clinical features associated with ID include fatigue, lassitude, pallor, weakness or palpitation, decreased work performance and sometimes headaches (Davies *et al*., 1973). The nature of these symptoms depends on the severity of the anaemia and the impact it has on quality of life (Cella, 1998; Wood & Ronnenberg, 2006). In childhood, measurable cognitive abnormalities may be observed (Pollitt, 1993). Another bizarre behavioural symptom that is peculiar to both children and adults is pica. Pica is characterised by the inappropriate ingestion of non-nutritive substances, although it may disappear following iron therapy (Moore & Sears, 1994). It is important to note that these symptoms are vague and may not necessarily provide adequate information for the diagnosis of ID in hospitalised patients (Elnicki *et al*., 1992). In general, the clinical symptoms of ID are somewhat non-specific, and are often difficult to attribute to anaemia. Findings common to anaemia may be present, and some individuals with ID may not even experience any symptoms. ID develops slowly in chronic blood loss and disorders in homeostatic mechanisms of iron (Frewin, 1997). As the clinical

manifestations of ID are usually unclear, most cases are mainly identified routinely by laboratory screening. High-risk groups, in which laboratory investigations have proved useful, include infants, children, adolescents and women between the ages of menarche and menopause (Dallman *et al*. 1980).

1.9 Iron deficiency anaemia

Iron deficiency anaemia (IDA) may occur as a result of occult chronic blood loss from the gastrointestinal tract, and its evaluation is always a very critical and challenging strategy for an effective diagnosis to be achieved. This common clinical problem must be properly evaluated and dealt with. It is understood that either acute or chronic blood loss is one of the numerous causes of IDA and both the upper and lower gastrointestinal tract may be the culprit of iron depletion (Peterson, 1989; Centre for Disease Control and Prevention, 2007). The examination of the colon has actually become a standard practice during investigation, and many clinicians would proceed to order colonoscopy or even barium-enema radiography regardless of signs and symptoms in the upper gastrointestinal tract. It has also been established that chronic colonic blood loss could be attributed to mass lesions (Peterson, 1989; Ahlquist, 1991). The symptoms that could be linked to the upper or lower gastrointestinal tract were traced to the presence of lesions in the

esophagogastroduodenoscopy or colonoscopy. Cases of cancer, gastric ulcer, duodenal ulcer, carcinoma, erotive gastritis and possible coeliac disease would represent conditions where the gastrointestinal tract should be investigated for possible bleeding.

1.10 Anaemia of chronic disorders

Anaemia of chronic disorders (ACD) is considered the second most prevalent type of anaemia caused by ID. This type of anaemia is seen in patients with acute or chronic immune activation, inflammatory or neoplastic diseases. It has some features that may be similar to IDA, and is also known as anaemia of inflammation (Weiss, 2002; Means, 2003). ACD is immune driven, and is characterized by inadequate production of erythropoietin, and causes an increase in the production of inflammatory cytokines (Means, 1995). This process will in turn increase the concentration of C-reactive protein (CRP) in the serum (Spivak, 2000). Even though the pathophysiology may not be fully understood, it is believed that the condition probably evolved as a cytokine-mediated defence against microbial pathogens leading to changes in iron homeostasis. This process ultimately leads to withholding of iron from microbes and from the erythroid precursors and the resultant effect is the subsequent limitation of the availability of iron for erythroid progenitor cells, and iron-restricted erythropoiesis (Jurado, 1997; Andrews, 1999).

Erythropoiesis can be affected by disease underlying anaemia of chronic disease by the infiltration of tumour cells into the bone marrow or by microorganisms, as seen in cases of human immunodeficiency virus, infection, malaria and hepatitis C (Stenvinkel, 2001). In ACD, like IDA, functional ID is the limiting factor of erythrocyte haemoglobinisation. This process will in turn lead to a reduction in erythrocyte and reticulocyte haemoglobin content, and the characteristic feature is the occurrence of hypoferraemia in the presence of reticulo-endothelial iron stores. Otherwise the supply of iron in IDA depends on the amount of iron stores, whereas in ACD, the supply relies on the rate of mobilisation. The consequence of this process is that, in ACD, functional ID may occur, even in the presence of adequate stores, when iron release is impaired, because iron is simply unavailable for delivery to the bone marrow, and thus erythropoiesis is compromised, thereby leading to anaemia (Fillet *et al*., 1989).

ACD is very common, and can be taken to be more common than any other type of anaemia except in cases where blood loss has resulted in iron deficiency (Means, 1999). It is without doubt that higher percentages of hospital patients will have ACD, and many studies have confirmed this fact. Cash and Sears, (1989) evaluated the anaemic patients admitted to a busy hospital over a period of 2 months. Patients with bleeding, malignancies and bleeding disorders were excluded from the study. It was found that 52% of

the total study population had ACD, using the standard laboratory diagnostic criteria.

Considering the types of anaemia and the extent to which they can cause damage if untreated, Brugnara *et al*., (1999) indicated that early recognition of subclinical ID is necessary to prevent systemic complications of the disease. Also, the recognition of IDA caused by lack of iron, malabsoption and bleeding should be identified, as these conditions do respond promptly to therapy (Ferguson *et al*., 1992). Although, it is not uncommon to see patients with certain inflammatory conditions, like inflammatory bowel disease, present with both ACD and IDA, in this group of patients the IDA is usually secondary to intestinal blood loss, and the diagnosis may point to inflammation rather than truly reflect the actual poor iron status (Weiss & Goodnough, 2005).

1.11 Haemochromatosis

Haemochromatosis (or HFE-related hereditary haemochromatosis) is simply described as iron overload with the presence or absence of tissue injury to the organs involved, manifested by cellular damage and fibrosis (Madra *et al*., 1996). Patients with iron overload absorb and store more iron than normal individuals. They continue to absorb more excessively even when they are overloaded. The excess iron is stored in the form of ferritin and

haemosiderin. These compounds are thought to be non toxic. However, iron released from storage sites can react with hydrogen peroxides to result in highly reactive oxygen species such as hydroxyl radicals. These can cause damage to proteins, nucleic acid and mitochondria (Madra *et al*., 1996). Some investigators considered haemochromatosis to be an inheritable disorder (McDonald, 1965; Saadi & Feingold 1974), and further investigation conducted over three decades ago corroborated the initial suggestion that haemochromatosis was transmitted as an HLA-linked, autosomal recessive disorder (Saadi & Feingold 1974). This was due to an inborn error of iron absorption. Although, the exact mechanism is unknown, it is believed that nearly 10% of the white Northern European origins are carriers of the gene indicating hereditary haemochromatosis. The prevalence indicates between three and five people out of a thousand are homozygous.

The gene responsible for hereditary haemochromatosis is known as HFE (*High iron* Fe). The protein product of the HFE gene is a 343-residue type 1 glycoprotein, which almost resembles the MHC class I protein in structure. Although changes in iron status appear to have little effect on the expression of HFE, the expression of the HFE gene is believed to participate in the metabolism of iron (Ganz & Nemeth, 2006). The potential linkage between the HFE gene product and cellular iron metabolism was discovered with the HFE protein forming a complex with the transferrin receptor (Feder *et al.,* 1996).

Two classic mutations associated with HFE are C282Y and H63D. The most common form of hereditary haemochromatosis is a G-to-A transition at nucleotide 845, which is indicated by (G845A). G845A results in a cysteine substitution at amino acid position 282, hence its designation as C282Y (Olynyk *et al.,* 2008). Some haemochromatosis patients also have an E-to-G transversion at nucleotide position 187 (C187G), this mutation is recognised as H63D (Olynyk *et al.,* 2008). H63D substitutes an aspartic acid for histidine and has an allele frequency of about 16% in the European population. H63D has been claimed to have little effect when inherited alone. However, it would contribute to the disease process when inherited with C282Y (Ganz & Nemeth, 2006).

HFE is thought to bind the transferrin receptor and subsequently influence the binding of transferrin and iron (Tf-Fe) intracellular delivery. HFE can indirectly influence the iron status of a cell population *in vivo* by modulating hepcidin expression (Figure 1.1). Some workers have shown that loss of HFE is associated with reduced expression of hepcidin, resulting in increased ferroportin-mediated iron efflux from duodenal enterocytes (Feder *et al.,* 1996; Frazer *et al.,* 2001; Ganz & Nemeth, 2006). This is an indication that HFE has a role to play in iron overload.

In patients with haemochromatosis, it is necessary to establish a diagnosis as soon as possible because death can occur within few years if untreated.

Treatment is by simple phlebotomy, removal of blood once or twice a week until about 50 L of blood has been removed.

Routine screening of haemochromatosis involves the assessment of iron status. The initial screening should include the measurement of serum iron, TIBC, TSAT and SF. If TSAT is greater than 60%, further investigations should be considered to diagnose haemochromatosis (Witte *et al*., 1996). Decreased transferrin synthesis may be associated with liver dysfunction, and consequently result in an increased level of TSAT. This has been linked to excessive alcohol consumption in some individuals (Tavill, 2001). Transferrin is a negative acute phase reactant, and may be decreased in inflammation. Also, SF is an acute phase protein which may be elevated in inflammation and malignancies. Therefore, the interpretation of SF in the diagnosis of haemochromatosis should take into account all the confounding factors in addition to TSAT.

Some workers have also found that the use of SF and TSAT together have good sensitivity and specificity of 0.85-0.90 and a positive predictive ability of 0.65 (Witte *et al*., 1996; Bassett *et al*., 1989). Individuals who have repeatedly higher TSAT, in addition to an elevated SF concentration, should be requested to undergo HFE genotyping. The diagnosis of hereditary haemochromatosis is confirmed if the haemochromatosis associated genotype (C282YC282Y or C282Y/H63D) is present. This study will evaluate the haemoglobinisation indices and compare with other haematological

parameters to determine their usefulness in the diagnosis and management of haemochromatosis patients.

1.12 Assessing iron status

Various attempts to arrive at a single assay that could accurately reflect iron status have failed. This is because the iron compartments of the body behave independently of one another and may not reflect the true behaviour of the biological conditions in the body at all times. This has caused irreparable damage to the individual patient, even in acutely anaemic patients, where one would expect that a simple measurement of HB would identify low iron status. It has on many occasions proved inaccurate, due to its low sensitivity and specificity (Cook, 2003). Even the determination of serum iron concentration has been made obsolete as a result of various biological rhythms. Therefore, a host of haematological and biochemical assays are used to identify the various stages of iron depletion that ultimately result in IDA.

Traditionally, scientists have used various biochemical and haematological tests to diagnose the presence or absence of ID (Worwood, 1997). The conventional iron storage parameter is SF (Briggs *et al*., 2001) and its use has been criticised by numerous workers because it is an acute phase responder and may be inaccurate in cases where inflammation is present (Briggs *et al*.,

2001; Thomas & Thomas, 2002). Even though the examination of stained bone marrow for haemosiderin is considered the 'gold standard' method, the technique is invasive and has been deemed unsuitable for the purpose of screening, especially in a large population setting (Hansen, 1983; Cook, 1994). Therefore, the non-invasive approach has been used for the evaluation of iron status. The biochemical indicators used include serum iron, SF, transferrin saturation, sTfR, and the ratio of these parameters have been studied in various pathological conditions to establish their usefulness. The haematological indices used are HB, HCT, MCV, MCH, abnormal RET-He and RBC-He indices have the ability to identify hypochromic or microcytic cells. The use of zinc protoporphyrin (ZPP) has also provided valuable indication for the diagnosis of ID and is still being used in some laboratories (Worwood, 1997). However, many of the parameters listed have their strengths and weaknesses. In most cases, the weaknesses have overpowered the diagnostic ability which makes them unreliable (Wish, 2006). Although some of these markers, including the RET-He and percentage hypochromic red cells show promise for identifying ID (Cullen *et al*., 1999; Chuang *et al*., 2003), none yet has been established to have the combination of accuracy, ease of use, cost-effectiveness, and widespread availability of the traditional tests.

Given these factors, the aim of this study was to investigate the diagnostic potential of new cytometric indices (reticulocyte haemoglobin content and erythrocyte haemoglobin content) in conjunction with the other

haematological indices, in assessing disturbances to iron metabolism. The focus of the remainder of this literature review will be to explore the available literature in describing these parameters of ID.

1.12.1 Haemoglobin

HB is the protein contained in red blood cells. It is responsible for the transport of oxygen to the tissues. Therefore, HB levels must be sufficiently maintained in the body to allow adequate tissue oxygenation.

HB determination is the most widely used screening method for ID. In general, the international agreement is that HB should serve as the key indicator of anaemia (International Nutritional Anaemia Consultative Group, 1977; World Health Organisation, 2010). HB in whole blood is expressed in grams per decilitres (g/dL). The normal range for males, is 14-18 g/dL, and females, is 12-16 g/dL (World Health Organisation, 2001). HB is measured by an automated cell counter from a tube of well-mixed ethylene diamine tetraacetic acid (EDTA) anti-coagulated blood. The principle involves the conversion of all forms of haemoglobin to the coloured protein cyanomethaemoglobin, the product of which is measured colorimetrically.

The World Health Organisation (WHO) defines anaemia as a haemoglobin concentration below the established cut-off levels. That is, below 13 g/dL in

men, below 12 g/dL in non-pregnant women and below 11 g/dL in pregnant women (World Health Organisation, 2001). HB concentrations are reduced to below-optimal levels in ID and, when the level falls below two standard deviations of the distribution mean in a normal population of the same gender and age, IDA is believed to be present. However, the definition of anaemia as suggested by the WHO expert committee and various other sources has been repeatedly decried, and attempts to propose a new lower limit of normal haemoglobin based on individual age, race and sex have been put forward (Cook *et al*., 2003; Beutler & Waalen, 2006). Artz and Dong (2008) challenged the suggestion of Patel *et al*., (2007) when they indicated that "alternative criteria are warranted" for defining anaemia in black individuals. The reason for the criticism was because the authors believed there could be serious consequences in lowering the threshold of the haemoglobin concentration in determining if a black individual is truly anaemic or iron deficient (Artz & Dong, 2008). The WHO criteria for using haemoglobin were established in 1968, and the threshold was believed to be based on a study involving a small population. Other findings over the last forty years based on large populations have highlighted the differences seen in haemoglobin concentrations in black, whites, young and old (Perry *et al*., 1992; Beutler & West, 2005; Beutler & Waalen, 2006). The survey carried out by the US National Health and Nutrition Examination Survey (NHANES III, 1988-1994) (US Department of Health and Human Services, 1994), and the Scripps-Kaiser database were based on large data sets, and the outcome of

these have been used to propose a new definition for anaemia. The National Health and Nutrition Examination Survey assessed a national population of 33,994 persons aged two months and older and seventy-seven percent of the people involved in the survey had their haemoglobin measured (Hollowell *et al*., 2005). The Scripps-Kaiser data also included the laboratory results of 41,038 adults (Beutler *et al*., 2002). The outcome of the two surveys predicts that more people would be classified as anaemic if the new haemoglobin obtained were to be adopted, even though it is closely related to those established by the WHO over forty years ago. Therefore, the WHO haemoglobin values still retain their approval in most laboratories and clinical settings today. However, based on the controversies surrounding the cut-off points of HB in its use for the definitive diagnosis of anaemia, it is right to say that HB is used for screening purposes only, because of its limitations, the complex relationship with health outcomes and to prevent misdiagnosis and misclassification of anaemia as a result of its low sensitivity and specificity (Cook, 2003). For example, in latent iron deficiency, a condition where even though the iron stores may have become depleted, the haemoglobin level will remain higher than the lower limit of normal (Verloop, 1970). The diagnostic criteria used for identifying ID vary between different studies and populations. Therefore, the interpretation of HB values becomes the responsibility of the ordering clinicians.

1.12.2 Ferritin: merits and demerits as a marker of iron stores

Ferritin is an iron-binding protein. It has been described as a ubiquitous protein with an iron core within a 24-polymer globular protein which is made up of heart and liver subunits, with molecular masses of 21 kDa and 19 kDa respectively (Harrison & Arosio, 1996). The cellular or tissue ferritin exists in two subunits as a polymer of heavier, more acidic H subunits and lighter, more basic L subunits (Cook, 1982; Theil, 1998). It is a spherical molecule consisting of an apoferritin shell and a ferric oxyhydroxide (FEOOH) crystalline core. The storage capacity of this protein shell may be up to 4500 molecules of iron, but is usually 2000 or fewer (Theil, 1998). In most cells, the source of stored intracellular iron is ferritin, and it is available in nearly all cells of the body. Ferritin is important detoxifying machinery produced intracellularly in response to an increase in iron content, to prevent free iron from forming reactive oxygen species. The synthesis of ferritin is influenced by the available iron, whereas in ID, ferritin synthesis is diminished. The release of iron from ferritin is probably non enzymatic, and may involve reduction by reduced flavin mononucleotide or other reducing substances. The released $Fe2^{+}$ leaves the crystal and diffuses out through a pore in the ferritin shell, hence the oxidation and reduction takes place rapidly (Torti & Torti, 2002). Ferritin is a very effective iron trap and also a readily available source of iron for metabolic purposes.

There is convincing evidence to suggest that serum ferritin concentrations are positively correlated with body iron stores, and it simply remains the best indicator of iron stores (Harrison & Arosio, 1996). Bates (1980) described serum ferritin as a sensitive and reliable measurement for the investigation of ID at an early stage. In the World Health Organisation and the Centre for Disease Control consultative group report in 2004, serum ferritin was chosen to be the best parameter for analysing the prevalence of iron deficiency in a population unless inflammation has taken over (World Health Organisation, 2004). It is also known as an acute phase protein, which can act as an indicator of inflammatory disease in malignancy. Elevated levels are also often seen in conditions such as hyperthyroidism, hepatocellular disease and cancer of the liver and pancreas. This is also seen during infection, where serum iron concentrations decrease opposite to elevated serum ferritin concentration (Lorrier *et al*, 1985), indicating that serum ferritin suppresses bacterial growth by segregation of iron from bacteria (Lipinski *et al*., 1991). Although the mechanism for its elevation in the solid tumour has not been fully elucidated, cytokines such as Tumour Necrosis Factor - α (TNF-α) and Interleukin - 6 (IL6) induce the H chain of ferritin structure. This suggests that the pathway related to inflammation is affected (Harrison & Arosio, 1996). However, the use of SF in the diagnosis of ID has been widely criticised, and many researchers have classed it as unreliable. This is because an inflammatory response triggers the release of the acute-phase reactant, and ferritin is therefore falsely elevated in patients with ID. The SF concentration

is frequently found to be elevated in relative proportion to iron stores. Various approaches have been suggested on how to utilise and interpret SF as an indicator of iron status. Some recommended measurement of SF with either the erythrocyte sedimentation rate and or CRP (Mast *et al*. 1998). The gender differences (normally lower in women) have also been discussed as an issue when evaluating its utility in the diagnosis of ID (Mast *et al*., 1998). However, an important finding in the report of the joint WHO/Centre for Disease Control and Prevention consultative group on assessing the iron status of populations suggests that a review of data should be carried out to examine the alternative approaches, by using one or two acute phase proteins, either jointly with serum ferritin or as a standalone index to address the controversies (World Health Organisation, 2004).

1.12.3 Serum iron, total iron binding capacity and transferrin saturation

ID results in a reduction in serum iron levels, an elevation in TIBC levels, and a net reduction in transferrin saturation. However, the diurnal fluctuations, diet and negative acute phase reaction have serious implications on the reliability of these parameters. There is marked overlap in these indices between normal and iron-deficient individuals. Normal or high serum iron may be seen in patients with ID. Concentrations can also decrease in late

afternoon or evening and then peak in the morning. The concentration of serum iron decreases when an acute phase response sets in, even in the absence of ID. The overlap diminishes the usefulness of indices in determining if an individual is iron deficient.

TIBC represents the amount of ferrous iron (Fe^{2+}) that can bind to transferrin in the blood (Fairbanks, 1999). TIBC is indirectly deduced from the measurement of the unsaturated iron binding capacity (UIBC). TIBC is increased in IDA, while a decreased value is seen in ACD.

Transferrin saturation (TSAT) is calculated using the following equation.

$$\text{TSAT \%} = \frac{\text{Serum iron concentration} \times 100}{\text{TIBC}} \qquad \text{(Eqn 2)}$$

TSAT still remains a very useful index in the diagnosis of haemochromatosis. TSAT < 15% indicates ID (Skikne, 1994), while TSAT > 55% may be indicative of iron overload. According to Professor Gleeson (personal communication, July 12, 2003) the recommendation is that TSAT > 50% in women and 55% in men should be followed by haemochromatosis genetic screening as it can be used to identify patients at risk of iron overload.

1.12.4 Zinc protoporphyrin

Zinc protoporphyrin (ZPP) is a metabolic intermediate that is produced in trace amounts during haem biosynthesis. This occurs following the incorporation of a zinc ion instead of a ferrous ion to form protoporphyrin IX, a precursor of haem (Labbe, 1999). The terminal pathway in haem synthesis is that iron is chelated by protoporphyrin. The reaction is enhanced by ferrochelatase, an enzyme that is bound to the mitochondrial membrane (Lange *et al*., 1999). The transfer of iron and zinc is tightly regulated, and both iron and zinc can compete for the metal binding sites on the ferrochelatase so that, when Fe^{2+} becomes suboptimal, Zn^{2+} is partially substituted (Lange *et al*., 1999; Outten & O'Halloran, 2001). This process results in increased ZPP formation (Labbe, 1999). Therefore in iron deficiency, ZPP is produced instead of haem, leading to an increased concentration. ZPP may be an indirect marker of inflammation as it will increase due to iron sequestration. Impaired iron utilisation is a common occurrence in ACD and will lead to an increased ZPP (Hastka *et al*., 1996).

Various workers have been studying ZPP since the 1930s. Van Den Bergh and Grotepass (1933) identified metal free protoporphyrin as contained in food. Following this discovery, other workers supported the evidence that truly protoporphyrin is responsible for the fluorocytes found in the circulating erythrocytes (Labbe, 1977). The presence of ZPP in the erythrocytes resulted in its deposition in the spleen and liver, therefore, when the aged

erythrocytes are sequestered, and the haem and ZPP are released, they become bound to the haem oxygenase, leading to slower degradation of haem (Maines, 1981).

Dagg *et al*., (1966) were the first to highlight the fact that erythrocyte protoporphyrin could be used as an indicator of ID. The interest later grew and the effort to identify protophophyrin compounds with the use of fluorescence technology was introduced by Lamola & Yamane in 1974. Since this discovery, many workers have been able to demonstrate that the presence of ZPP in circulating erythrocytes was linked to exposure to lead (Lamola, 1974).

The potential role of ZPP levels in ID diagnosis have been explained by Hastka *et al*., (1992) and some other previous workers (Crowell *et al*., 2006). They reported significant correlations between ZPP and ID. Elevated ZPP levels are an indication of iron-deficient erythropoiesis and, in a normal person without ID, ZPP is demonstrated to be < 60 µmol/mol haem. In their study, Mei *et al*., (2003) showed that erythrocyte protoporphyrin is a better screening parameter for ID than haemoglobin, and believed ZPP to have equal sensitivity and specificity with that of HB for identifying ID. Studies have shown that a slight shift from iron to zinc during haem biosynthesis will have a huge impact on the ZPP/H ratio in circulating erythrocytes and reticulocytes (Labbe, 1977). Due to the fluorescent technology employed in the measurement of ZPP, it is readily detectable as iron deficiency, even in its

lowest amount when present in the circulating erythrocyte, even before a lower level of HB is detected. This demonstrates that changes in ZPP are remarkably important in the diagnosis of ID (Brugnara *et al*., 1999). The World Health Organisation/Centre for Disease Controls (2004) report also suggests that meta-analysis of data should be looked at to examine the threshold of ZPP as an indicator of iron status. Seven years after, most of these recommendations have not been met.

1.12.5 Transferrin receptor

The transferrin receptor is a transmembrane protein whose function is to mediate the cellular uptake of iron from transferrin, a plasma protein which helps to transport iron in the circulation (Beguin, 2003). The expression of transferrin receptors by cells is proportional to iron requirements, especially for erythropoietic activity (Beguin, 2003). The process of uptake involves the binding of transferrin to the transferrin receptor. It is believed that the transferrin receptor does not directly interact with iron, but it is involved in iron acquisition by most cells, and part of a complex system that has evolved to control iron uptake and storage (Ponka *et al*., 1998).

Jandl *et al*., (1956) were the first to suggest the existence of specific receptors for transferrin on the reticulocyte membrane, but it was not until the 1970s when several laboratories carried out further work and were able

to isolate the transferrin receptor from reticulocyte membranes, and subsequently from placenta and cultured cell lines (Hu & Aisen, 1978; Witt & Woodworth, 1978; Enns & Sussman, 1981).

The human form of the transferrin receptor was first identified and characterised by Trowbridge and Omary in 1981. It consists of two identical transmembrane sub-units, with a molecular weight of 84,910 Da, containing 760 amino acid residues, which are joined by two disulphide bonds at cysteine 89 and 98 (McClelland *et al*., 1984).

Another determinant of iron status that has recently joined other biochemical ID diagnostic profile is the measurement of soluble transferrin receptors (sTfR). This parameter is typically reserved to distinguish between IDA and ACD, where ACD is associated with chronic inflammation and neoplasia (Rimon *et al*., 2002). The findings of the study conducted by Chua *et al*., (1999) in the investigation of the role of sTfR in ID and ACD in the elderly population concluded that there is possibility that cases of ID and IDA could be missed as a result of complexities of clinical conditions and inflammation. The sTfR test is based on the fact that erythroblasts in the bone marrow will increase the presentation of membrane transferrin receptor in cases of ID. The concentration of circulating sTfR correlates with the total receptor content of the cells (Cook, 1999).

The sTfR level remains unchanged in ACD, which is the reason why some workers have approved its uniqueness in distinguishing between iron deficiency as a result of various pathological conditions or inflammation from every other type of IDA (Ferguson, 1992). Elevated sTfR occurs earlier in functional iron deficiency, even sooner than the changes observed in free erythrocyte protoporphyrin or mean cell volume (MCV) (Cook & Skikne, 1989; Punnonen & Irjamaki, 1997).

In the study performed by Dimonte *et al*., (2002), they investigated the use of sTfR in assessing ID without anaemia in a group of patients who donated preoperative autologous blood. The findings showed sTfR to be a reliable and sensitive marker in the detection of ID (Dimonte *et al*., 2002). Another important related finding by Flesland *et al*., (2004), demonstrated sTfR as a valuable assay, and argued it could be better than ferritin in the diagnosis and prevention of ID in blood donors.

The sTfR concentrations are determined by an immunoassay technique. This technique provides an alternative to the very cumbersome ferrokinetic studies that were previously used. In contrast to SF, sTfR levels are not elevated in patients with acute infections, including hepatitis, chronic liver disease and other conditions with chronic diseases, except where there is a co-existing ID (Ferguson, 1992). A reduced level of sTfR is observed in aplastic anaemia and renal cases when erythroid cell mass is reduced and in hyperproliferative states (Worwood, 2002). The shortfall of the sTfR assay is

that it lacks standardisation. Even though there are several commercial assays available, there is no common reference material available for its validation (Brugnara, 2003).

1.12.6 New diagnostic dimension in the investigation of iron status

It is clearly understood that iron balance is controlled by the rate of erythropoiesis and the size of the iron stores. This indicates that, in IDA, the supply of iron depends on the iron stores, whereas in FID, the supply depends greatly on the rate of mobilisation of iron. The production of 2-3 million red blood cells every second requires an adequate supply of iron which will allow the developing red cells to synthesize sufficient haemoglobin (Fillet *et al*., 1989). Therefore, FID is defined as an imbalance between the iron required for erythropoiesis and the iron supply, which is not maintained at a sufficient rate to allow normal haemoglobinisation of red cells and the reticulocyte, even though there is an adequate body iron store (Frewin *et al*., 1997; Ponka & Lok, 1999). This situation will lead to reduced reticulocyte haemoglobin content (RET-He) and red blood cell haemoglobin content (RBC-He), and this scenario is typically seen in patients with ACD (Fillet *et al*., 1989, Thomas & Thomas, 2002).

The uncomplicated cases of ID can be diagnosed using the traditional haematological and biochemical markers to a certain extent and have been

described within this literature review. However, in complicated cases, the results of these traditional parameters may not be helpful. Even when the clinical manifestations are subtle, the identification of deficiency is still a worthwhile exercise, especially in the monitoring of treatment.

The complicated case mentioned here is FID in acute or inflammatory conditions, because most patients go on to develop chronic anaemia. These types of anaemia pose a huge challenge to scientists and physicians when the goal is to diagnose and then initiate treatment.

1.12.7 Reticulocyte haemoglobin and red cell haemoglobin content

Reticulocyte haemoglobin (RET-He) and red blood cell haemoglobin content (RBC-He) have been known for nearly a decade. The parameters have acquired great clinical interest and importance in the assessment of FID. This is because the reticulocyte is the earliest form of erythrocyte released from the bone marrow into the peripheral blood. Reticulocytes actively produce haemoglobin, where the matured red blood cells do not. They circulate for 18-36 h prior to maturation. Therefore, the haemoglobin concentration is measured in the reticulocyte, because they represent a real-time and direct determination of the current status of erythropoiesis and indicate the amount of iron contained in the red cell precursors (Brugnara *et al*., 1999; Brugnara, 2000; Thomas & Thomas, 2002).

The use of haematological parameters to identify FID was originally described by MacDougal (1992). He proposed that patients with FID could be identified by the use of % hypochromic red blood cells which was uniquely available on the Bayer H* haematology analyser at the time. The measurement of this index gained massive popularity amongst the renal physicians within Europe and the United Kingdom at that time and, shortly after that, literature began to present those parameters that measure the characteristics of the reticulocyte. The prospective indicator explored in many of those studies was the use of CHr, which was also unique on Bayer (H*3 and ADVIA). CHr is known as the mean value derived from the haemoglobin content of an individual reticulocyte, which is measured by the light scatter in the Bayer instrument.

Until recently, the most widely studied reticulocyte indices were reticulocyte haemoglobin content (CHr) and the portion of hypochromic red cells (%HYPO) which were restricted to the Bayer ADVIA 120. However, with the introduction of Sysmex XE2100^{TM} ten years ago, two groups (Briggs *et al*., 2001; Thomas & Thomas, 2003) investigated the potential benefits of identifying FID using reticulocyte haemoglobin content (RET-He). The findings were that RET-Y provided good correlation to CHr, sTfR and % hypochromic red cells, and that RET-He could be an acceptable parameter for indicating FID. They also suggest the parameter may be substituted for CHr in patient management (Buttarello *et al.,* 2004). Luo *et al*., (2007) also compared the

diagnostic efficacy of CHr to other markers of ID in Chinese premenopausal women. They recommended further studies to determine whether CHr should be the preferred screening tool in the early detection of ID in a larger and unselected population of premenopausal women and children.

The Sysmex XE-2100 (Sysmex, Kobe, Japan) is a discrete automated haematological analyser which utilises new technology to improve the quality of cell analysis, including red cell counts, immature granulocyte count and an optical fluorescent platelet count in situations where an impedance count is unreliable (Briggs *et al*. 2001). It uses automated fluorescent cytometry with the aid of polymethine dye to measure reticulocyte haemoglobin equivalent. RET-Y and RBC-Y values are generated from the reticulocyte channel of the Sysmex-XE2100 analyzer using the same forward scatter and fluorescence technologies that provide reticulocyte counts and maturation parameters (Brugnara, 2000). RET-Y is the forward scatter of fluorescence-labelled reticulocyte haemoglobin equivalent (RET-He), and RBC-Y represents the forward scatter of fluorescent-labelled erythrocytes which can be represented as RBC-He. RBC-Y and RET-Y indices are determined in a manner that is technically different from those on the Bayer Advia 120 analyzer. Therefore, they consequently require investigation to determine how much information they can provide to accurately diagnose and monitor the treatment of ID. This study is concerned with investigating these parameters

(RET-He and RBC-He), either as stand alone or in conjunction with other markers of iron that have been previously described.

A number of studies have been conducted, but not in a similar patient population as proposed here. The majority of the studies already carried out focussed on renal patients undergoing haemodialysis. Two studies examined the clinical utility of the reticulocyte index (CHr), as measured on the Bayer Advia 120 and 2120, with comparison to other markers of ID. The optimal CHr concentration cut-off values for the diagnosis of ID differed slightly at 26.0 and 27.5 pg in both studies (Brugnara, *et al*., 1999; Ullrich *et al*., 2005).

In a number of clinical studies, CHr has proven to be useful in differentiating anaemia and, in monitoring erythropoietin therapy in renal patients undergoing dialysis, the sensitivity was good for detecting relative ID, with a false positive of 20% (specificity 80%). The study carried out by Luo *et al*., (2007), compared the diagnostic efficacy of CHr to other markers of ID in Chinese premenopausal women. They recommended further studies to determine whether CHr should be the preferred screening tool in the early detection of ID in larger and unselected population of pre-menopausal women and children.

Most of the studies carried out to examine the clinical utility of RET-He mainly focussed on the treatment and monitoring of patients with renal disease undergoing dialysis. Most of the findings have indicated that studies

would be required in a large population to determine how much information could be gleaned. This study would however, consider other disease conditions and, more importantly the hospitalised patients to be able to identify functional iron deficiency (FID) in disease process especially in cancer and inflammation.

RET-He is a new parameter which provides direct evaluation of the bone marrow activity. It mirrors the balance between the demand and supply of iron for erythropoiesis. RET-He is easily measurable and has gained merit in the diagnosis of FID especially in patients with end-stage renal failure and in cancer – related anaemia (Franck *et al.,* 2004). The mean cellular haemoglobin content of the reticulocyte (CHr) parameter provided by the ADVIA 120 and 2120 directly reflects the recent haemoglobin synthesis in bone marrow precursors. However, CHr remains far reaching for many laboratories, which is a major disadvantage. Also, the exact threshold values have not been established. The cut-off values used for CHr vary from 26 – 32 pg, depending on the laboratory and the analyser used. This would invariably lead to lack of standardisation. It has been agreed that 29 pg should be the exact cut-off value for RET-He, to define deficient erythropoiesis (Miwa, 2010).

Although studies examining the diagnostic usefulness of various haematological and biochemical parameters have been undertaken, no single study of iron status with inflammation have so far included all the

measurements of MCV, SF, MCH, RDW, ZPP, Fe, sTfR, RBC-He and RET-He indices. Therefore, this study will incorporate all the haematological and biochemical parameters and include RET-He and RBC-He.

1.12.8 Peripheral blood smear

Microscopic examination of peripheral stained blood is used to supplement the information obtained from the haematology analyser or cell counters in the assessment of iron status. The accurate identification of stained red blood cells and white blood cells is done by experienced scientists. IDA is recognised from a combination of abnormal iron supply studies and microcytic hypochromic red blood cell morphology (Hillman, 1998). Anaemia can be classified into three groups depending on the morphology of the red blood cells.

- Microcytic hypochromic features in the smear indicates a decreased MCH and MCH concentration of the red blood cell. The morphology in the smear would show an expanded central zone of pallor. The small hypochromic red blood cells known as microcytes are typical findings in IDA, ACD or alpha and beta thalassaemia (Figure 1.3) (Hillman, 1998).
- Normocytic normochromic, in acute haemorrhage, haemoglobinopathies and HIV related anaemia and ACD.

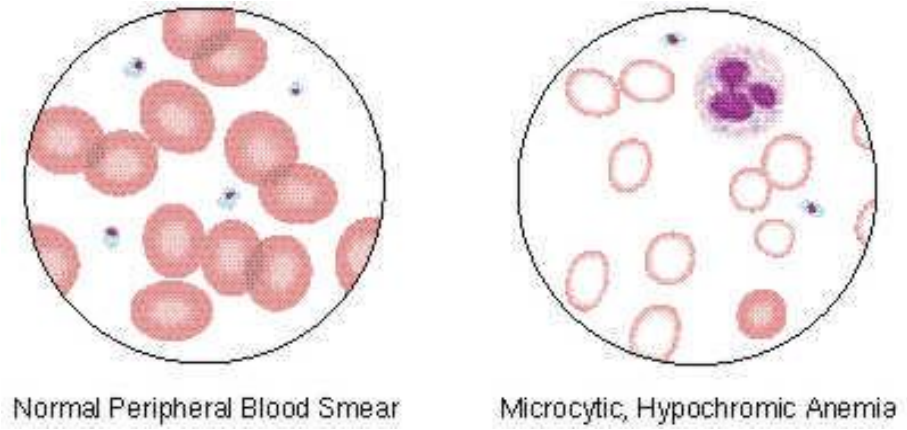


Figure 1.3: Blood smear – normal, microcytic and hypochromic.

- Macrocytic features in the smear are indicated by large oval red blood cells usually > 8.5 mm with an increased MCV usually around 120 fL, with normal MCH. Likewise, round oval macrocytic cells are slightly larger than normal, and the MCV is usually > 95 fL. The presence of these cells is indicative of impaired bone marrow DNA synthesis from chemotherapy or other inherited disease. Possible causes include liver disease, megaloblastic anaemia, B12 or folate deficiency, haemolytic anaemia, consequence of reticulocytosis and aplastic anaemia (Figure 1.4).

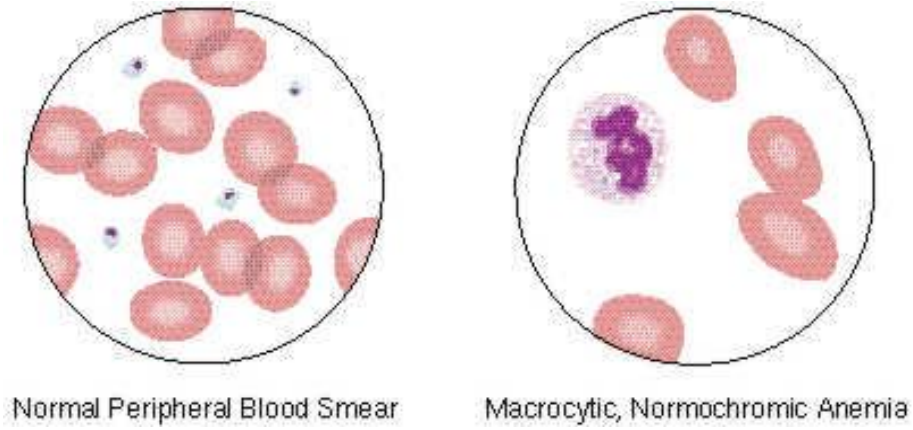


Figure 1.4: Blood smears – normal, macrocytic and normochromic.

The automated analysis of the blood cells is mostly accurate, reproducible and readily available. The value of the use of blood smears in detecting the deviation of cells from normal size has decreased, even with the experienced haematologists (Dallman, 1984). Jen *et al*., (1983) concluded that the examination of blood smears provides no additional information to the indices of patients with microcytic anaemia. Apart from the fact that an experienced microscopist is required to report on the cells, the disadvantages of non-uniformity of cells, possible over-staining and under-staining present issues for consideration.

1.12.9 Mean cell volume and mean cell haemoglobin

Mean cell volume (MCV) indicates whether a RBC is smaller than normal (microcytic), or larger than normal (macrocytic). IDA manifests itself as a

microcytic, hypochromic anaemia. Microcytic anaemia, generally defined as a MCV < 80 fL, also shows a reduced mean cell haemoglobin (MCH). This may be observed either prior to or alongside the haemoglobin reduction (Bolton-Maggs & Thomas, 2003). MCV represents the average volume of a single erythrocyte; its reduction can indicate developing ID in overt anaemia. MCV measurements can be done accurately by the use of an automated haematology analyser with impedance or optical light scatter. The British Society of Gastroenterology recently revised the guidelines for the diagnosis and management of IDA in the general population, and MCV was listed as one of the reliable parameters in the diagnosis of IDA in the presence of chronic inflammation (Jolobe, 2000).

It has been suggested by various workers that low MCH correlates better with low ferritin levels. Low MCH is therefore considered better than low MCV in identifying patients with IDA, because it is less influenced by the cell counter and by prolonged storage conditions (Jolobe, 2000). Despite this understanding, MCH is still not widely used in routine clinical practice (Jolobe, 2000; O'Broin *et al*., 1990). Although ID is typically characterized by a MCV and MCH of < 80 fL and < 26 pg, respectively (Bainton & Finch, 1964), it is still a matter of controversy to determine which of the two parameters has greater predictive value due to the associated changes in chronic diseases and inflammation (Cook, 2003). Decreased MCH hypochromia and increased percentage of hypochromic red cells, and reduced MCV increases the

sensitivity and specificity when microcytosis, hypochromasia and pencil cells are demonstrated (Jolobe, 2000).

To calculate the MCV, expressed in femtoliters, the following equation is used:

$$MCV = \frac{HCT\ (\%) \times 10}{RBC\ counts\ (millions/mm^3\ blood)} \qquad \text{(Eqn 3)}$$

To calculate the MCHC, expressed in g/dL, the following equation is used:

$$MCHC = \frac{HB\ (g/dL) \times 100}{HCT\ (\%)} \qquad \text{(Eqn 4)}$$

1.12.10 Acute-phase proteins

The acute phase response is the innate, physiological and biochemical response to the stress of injury, surgery, trauma, inflammation, neoplastic growth, immunological disorders or infection. It is denoted by higher plasma or serum acute-phase proteins (APPs) (Gitlin & Colten, 1987; Maes *et al*., 1997). The role of the acute phase response is to prevent further damage to the tissues affected by proteases produced by the pathogens and to initiate

the repair process, which is done by modulating T-lymphocyte function and the complement system, and also initiation of the inflammatory process (Gitlin & Colten, 1987; Mackiewicz *et al*., 1991). The process of production of the APPs is promoted by several intercellular signalling systems, of which the most important is the inflammation-associated cytokines, like IL-6, which can stimulate APPs in response to varied stimuli. Some of the APPs include C-reactive protein, α1-antitrypsin, haptoglobin, α1-acid glycoprotein and fibrinogen.

The understanding of the role of APPs in iron status is particularly important to allow clinicians to identify any underlying conditions which may prevent the treatment of the ID. For example, the role of hepcidin, an iron-regulated acute-phase protein, has been implicated in iron homeostasis and anaemia of chronic disease, because its increased production in iron overload and inflammation is modulated by IL6 and inhibited by TNF- α (Nemeth *et al*. 2004). Therefore, in ID, low serum iron and elevated SF in the face of an acute phase response indicates some underlying inflammatory condition. Bayne *et al*., (1996) studied the serial changes of serum iron, TIBC, SF and CRP, and some other acute phase proteins in various illnesses. They found elevated SF appeared in line with high CRP measured in rheumatoid arthritis, tuberculosis and sepsis, which suggested that ferritin acted as an acute-phase protein, especially in those cases with underlying iron status. The presence of acute phase protein has been widely reported in many types of cancers, and

this obviously interferes in iron metabolism in this group of patients (Denz *et al*., 1992). In the study carried out by Feelders *et al*. (1998), samples were taken from inoperable patients with sarcoma or melanoma for the measurements of acute phase protein, elevation in CRP and SF were found to parallel each other. CRP level reportedly peaked 4 h after the initiation of an inflammatory process.

The joint WHO/CDC committee recognised the need to consider data on the relationship between SF, sTfR and different acute phase proteins to assess and determine which best correlated with SF in the face of inflammation. The recommendation was that measurement of one or more acute phase proteins e.g. C-reactive protein (CRP) and α1-acid glycoprotein could be used, being the most frequently used index of inflammation and one that could predict changes in the ferritin concentration due to inflammation as inferred by many previous workers (WHO/CDC, 2004; Gabay & Kushner, 1999).

1.12.11 C-reactive protein

C-reactive protein was discovered in 1930 when researchers described a third serologic fraction, or “fraction C” that could be isolated from patients infected with *Pneumococcus* that was different from the formerly identifiable polysaccharide and nucleoprotein fractions detectable by specific antibody response using immunological techniques (Tillet & Francis, 1930). Another

decade later, CRP was described as an “acute phase reactant” and was elevated in patients with inflammatory stimuli, like myocarditis and inflammation associated with rheumatoid arthritis (Abernathy & Avery, 1941; Macleod & Avery, 1941). Many previous workers have identified CRP as being produced in hepatocytes, and also indicated that an increase in CRP is linked to myocardial infarction and ischaemia (Pepys *et al*., 1978; Baltz *et al*., 1982). CRP is present in the serum of healthy individuals with a value less than 1 mg/dL. Values above this may indicate some sort of systemic inflammation. Up regulation of this protein has also been linked to a rise in interleukin 6 (IL6) and, to a lesser extent, by tumour necrosis factor TNFα (Darlington *et al*., 1986). As CRP plays an important role in the innate immune cascade, it is important to evaluate its role as a marker for detecting the level of inflammation in ACD.

1.12.12 Serum hepcidin

The antimicrobial protein known as hepcidin has been identified and implicated to be a key regulator of iron absorption and mobilisation. Hepcidin is produced in the liver and secreted into plasma and cleared by the kidneys. It causes a decrease in iron release from its stores by down regulating the expression of ferroportin (Park *et al*., 2001; Pidgeon *et al*., 2001, Ganz, 2006). Its synthesis is a result of iron overload and inflammatory stimuli such as

interleukin-6 (IL-6) and lipopolysaccharides (Kemma *et al*., 2005). Decreased hepcidin is seen in iron deficiency, accelerated erythropoiesis and hypoxia (Nicolas *et al*., 2002). Although advances in the understanding of the pathophysiology of anaemia of chronic disease, including the disturbances of iron homeostasis, impaired proliferation and even response to iron therapy have been made, to date the bulk of the information has been drawn from animal experiments, and the few human studies available were based on *in-vitro* experiments using human sera. Therefore, detailed association between erythropoiesis and hepcidin has not been fully elucidated in clinical settings (Kanda *et al*., 2008). The study carried out by Dallalio *et al*., (2003), to assess the correlation of hepcidin to anaemia and to clinical iron metabolism showed that hepcidin does not correlate as well with ACD. They concluded that a better understanding of this protein is required to give some reliable information in the diagnosis of ACD.

1.13 Study hypothesis

Although many studies have been performed to examine the diagnostic usefulness of various haematological and biochemical parameters as described above, no single study has so far incorporated the measurements of MCV, SF, MCH, RDW, ZPP, sTfR, Fe, RET-He and RBC-He indices together. Therefore, clinical interest should be focused on the early diagnosis of ID and

IDA to prevent and manage systemic breakdown and complications. The focus of this study is to evaluate the potential benefits of integrating the cytometric indices (RET-He and RBC-He) into the analytical repertoire for the evaluation of iron status.

The aim of this study is to evaluate the cytometric indices RET-He and RBC-He in conjunction with the other haematological parameters in the assessment of iron status in hospitalised patients.

1.14 Research aims and objectives

The overall aim of the research was to investigate the diagnostic potential of new cytometric indices (RET-He and RBC-He) in conjunction with other haematological indices, in assessing disturbances to iron metabolism.

- To evaluate the stability and reproducibility of the RET-He and RBC-He, and to assess their ability to measure accurately low iron status in patients.
- To investigate the diagnostic potential of the currently used haematological and biochemical screening procedures for detecting patients with ID, and to compare these to the erythrocyte and reticulocyte haemoglobin content indices.

- To determine whether the haemoglobinisation indices may be used diagnostically to preclude the need for other biochemical assessments of iron status. (Note: the study will also address the comparative diagnostic value of these indices in the investigation of inflammatory conditions which are prevalent in hospitalized patients and selected haemochromatosis patients).
- To evaluate the potential values of erythrocyte and reticulocyte haemoglobin content indices and compare with other haematological parameters in assessing how patients with haemochromatosis respond to treatment.
- To suggest guidelines for the diagnosis of ID by considering the predictive ability of the initial iron screening tests and the added value of more specialised and costly tests.
- To analyse the results and discuss findings.
- To draw conclusions and make recommendations for change of practice and make suggestions for further studies if required.

CHAPTER 2 Materials and methods

2.0 Overview and study design

The main aim of this thesis was to investigate the diagnostic potential of the new cytometric indices, the reticulocyte (RET-He) and erythrocytes haemoglobin (RBC-He) contents in conjunction with other haematological indices, in assessing disturbances to iron metabolism.

The study is a broad assessment of the usefulness of RET-He and RBC-He in determining how much information could be gleaned to improve further the assessment of iron status in hospitalised patients.

The research was therefore conducted following different stages of investigation and evaluation. In order to overcome the majority of possible issues that are likely to be associated with a study such as this, the evaluation of different pre-analytical and standard variables were considered at the beginning of the study.

2.1 Investigative method

The method of investigation employed in this research followed the established protocols in the haematology and biochemistry sections of the clinical pathology laboratory at St James's Hospital, Dublin, Ireland.

Considering the advantages and disadvantages of the methods currently in use, the author adapted other analytical methodologies which could ultimately lead to a better turnaround time and an improved protocol in the assessment of iron status.

2.1.1 Sample collection

A total of eight hundred samples were obtained for this study, of which eighty-nine were from non-anaemic adults to serve as controls to establish threshold levels in the parameters tested. Six-hundred and eleven samples were from hospitalised patients and one-hundred samples were from previously diagnosed haemochromatosis patients currently undergoing treatment.

All samples were drawn by the phlebotomists from the cubital vein using the standard vacutainer blood collection system. Subjects were requested to sit upright on the chair and their arm was supported to prevent movement. A tourniquet was applied 5-8 cm above the venepuncture site. The site was swabbed with a 70% alcohol disposable wipe, which was left to air dry for about 20 s prior to venepuncture.

One K_3EDTA blood and two clotted samples were taken from all participants. Blood samples drawn into tripotassium EDTA tube and Plain Sarstedt bottles were adequately labelled and were used for the analysis.

2.1.2 Control group

Eighty-nine (11.1%) samples were collected from non-anaemic adults. The absence of any systemic inflammation was confirmed in the normal group by the value of C-reactive protein (CRP).

2.1.3 Patient group (hospitalised)

Six-hundred and eleven (76.4%) samples were obtained from hospitalised patients at St James's Hospital Dublin. Cases included in this group were symptomatic and asymptomatic conditions, such as gastroenterological disease, renal disease, cancer, rheumatoid arthritis and other abnormalities. Children and pregnant women were not included in this study. Sample selections were sequentially done from the residual blood samples based on the clinical diagnosis and information provided by the haematology consultants. The author ensured that individual diagnosis provided on the request form fulfilled the criteria for sample selection for the study.

2.1.4 Haemochromatosis group

One hundred (12.5%) samples were received from haemochromatosis patients currently undergoing treatment. These patients were previously diagnosed with the haemochromatosis genetic disorders (HFE). The patients also participated in the study as part of the National Surveillance control for haemochromatosis patients. Only one tripotassium EDTA blood sample was taken for the measurement of the full blood count. The purpose was to determine whether RET-He and RBC-He indices could be used to monitor treatment and management of the haemochromatosis patient group. A decision was taken not to measure serum ferritin on the samples, because patients have had serial ferritin and iron measurements over the period of diagnosis and treatment, and results were observed to be very high, therefore, no new information could have been provided.

2.1.5 Ethical approval

The study was conducted after approval was obtained following an application to the research ethics committee of both St James's Hospital and the Adelaide and Meath Hospital (Appendix A). Patients recruited in this study were not personally consulted as the blood samples used were requested by the clinicians for clinical investigation rather than specifically for research purposes. The added investigations included RET-He, RBC-He, ZPP,

CRP and sTfR. For this reason, patient consent was not required as indicated in the ethics approval documentation.

2.1.6 Power calculation and sample size

The sample size selected for the study was based on a power calculation related to the degree of statistical changes in iron status parameters in the early pilot study undertaken, evaluating the RET-He and sTfR in the differential diagnosis of anaemia of chronic disease from iron deficiency anaemia (Appendix I). The author utilised the outcome of the findings with regards to the differential diagnosis between ACD and IDA, and used it to detect how many samples were needed. Statistical power (also called a power analysis and typically set at 0.80) is the probability of finding statistical differences in the data set.

The number of samples required with 80% chances to identify ID and IDA using RET-He and RBC-He in hospitalised patients with chronic disease, inflammation and rheumatoid arthritis, and assuming a two-tailed test at 5% significance level. The following results were obtained:

1). Female: Pilot study results suggested a mean change of 6, with standard deviation 28. With a 5% significance level and 80% power, 344 subjects were required to show a significant outcome.

2). Male: Pilot study results suggested a mean change of 6, with standard deviation 23 for male population. With a 5% significance level and 80% power, 258 subjects were required to show a significant outcome.

The power calculation suggests that a total of 602 samples (male and female) would be required in order to achieve significant outcome. A total of 611 samples were obtained from hospital patients with cancer, rheumatoid arthritis, gastroenteritis and others with inflammatory response. The increase from 602 to 611 was necessary to prevent any inadequate or unsuitable samples that may have resulted in not recruiting enough. 611 samples were required to show a significant relationship between male and female in the hospitalised patients. The margin of error was also considered in the sample size calculation.

2.1.7 Identification and classification into disease categories

Clinical details provided on the database of the Telepath laboratory information systems (LIS) were used for identifying the patients. Patients' records were matched accordingly to the information which was available under the password protected LIS.

Patients were anonymised in agreement with the statement submitted to the ethics committee. Patient age and the laboratory investigations performed

were the only data available within this research. To establish the correct diagnosis, presenting signs and symptoms were considered to allow for the true classification of disease diagnosis.

2.2 Methodology and principles of analysis

2.2.1 Full blood counts using flow cytometry methods on the Sysmex XE-2100TM

The Sysmex XE-2100^{TM} is the haematology analyser used in this study for the full blood count (FBC) analysis. The analyser uses fluorescence flow cytometry and hydrodynamic focusing technology to measure the standard five part differential, immature granulocytes (metamyelocytes, myelocytes and promyelocytes), nucleated red blood cells (NRBC), reticulocyte count, immature reticulocyte fraction and "optical" fluorescent platelet count. The combination of side scatter (inner cell), forward scatter (volume) and fluorescence intensity of nucleated cells has brought an improvement to how normal and abnormal cells are classified, thereby removing the need for manual counting. The performance evaluation of this analyser, as evaluated by some workers, found there was excellent correlation in linearity, and the reproducibility produced good results with insignificant carryover, when

compared with results obtained from Sysmex SE 9500^{TM} and the manual counting (Briggs, *et al*., 2001; Brugnara *et al*., 2006).

RET-He and RBC-He are available as an optional feature on the analyser, it measures the incorporation of iron into the red cell to assist in anaemia evaluation and management. FBC were performed according to the established standard operating procedures in the haematology laboratory, and in accordance with the Sysmex-XE2100 (Kobe, Japan) manufacturer's instruction. The methods were optimised to allow further cytometric analysis of the reticulocyte and erythrocyte haemoglobin measurements.

The Sysmex XE-2100^{TM} analyser used throughout this study was the same analyser routinely used in the haematology laboratory of St James's Hospital, Dublin. The analyser was calibrated and controlled at the beginning of the study. Reagents were loaded and replenished as required on the analyser for the purpose of this project.

Reagents for erythroblast measurement are Stromatolyser-NR, lytic agent and staining liquid. The surfactant contained in the Stromatolyser-NR lytic agent lyses the erythrocytes, exposes the erythroblast nuclei, and uses the leucocyte membranes. This agent is used to distinguish erythroblast from leukocytes, especially the lymphocytes. The technology uses proprietary polymethine dye, which acts by penetrating the cells and staining the nucleic acid of the cytoplasmic organelles.

Reagents for white blood cell (WBC) 4-differential counting known as Stromatolyser-4DL, Stromatolyser-4DS were loaded on the Sysmex XE2100^{TM} analyser accordingly. The surfactant contained in Stromalyser-4DL lyses and dissolves erythrocytes and platelets. Following this process, the dye contained in the Stromatolyser-4DS enters the WBC, and allow the staining of the nucleic acid and cytoplasmic organelles, such as the endoplasmic reticulum, to take place.

The following steps were undertaken using the manual mode to measure full blood counts on the Sysmex XE-2100^{TM} with the incorporation of the reticulocyte parameters.

- To analyse an EDTA blood sample, the manual option was used on the analyser. Sample barcode numbers were scanned using the hand held barcode scanner. EDTA samples were presented to the sample probe on the analyser in the order in which they were obtained. The sample was removed and the manual rinse cup lowered to wash and dry the sample probe.
- Internal quality controls (IQC) were treated the same every time a measurement was performed to ensure reliability and reproducibility.
- Results obtained were displayed on the Sysmex XE-2100^{TM} LCD screen. A sample of the printout is presented in the Appendix E. The controls were checked visually to ensure every parameter was within

the range originally configured, and 'OK' was pressed. This action sent the patient's result to the laboratory information system.

2.2.2 Stability studies procedure

Stability studies were undertaken to assess the effect of prolonged storage at different temperatures on the following parameters, RBC-Y, RET-Y, MCV, MCH and HB. The Sysmex XE-2100^{TM} was used for the measurements of K_3EDTA blood samples to assess the effect of storage at temperatures of 4^{o}C, 20^{O}C and 30^{o}C for a period of 96 h. Fifteen K_3EDTA samples were selected for this study, (normal and abnormal samples). All samples were tested at baseline when received in the laboratory and up to 96 h in order to assess changes over time.

Individual K_3EDTA samples were aliquoted into three plain sample tubes capped, and labelled according to the information contained on the primary samples. Barcode labels were also given to each sample and the corresponding form to allow easy tracking and identification. Tubes containing equal sample volumes were stored at regulated temperatures of 4^{o}C (refrigerator), 20^{o}C and 30^{o}C (water bath). The temperatures were monitored by the use of thermometers and any sudden changes in the refrigerator temperature were monitored centrally using the pathology temperature monitoring systems. Serial measurement for RBC-Y, RET-Y, MCV, MCH and HB on the stored samples were performed over a period of four

days. Measurements were performed at the same time on each day to allow for equal storage conditions.

2.2.3 Ferritin assay using chemiluminescent technology

Serum ferritin was analysed on the Beckmann Coulter Access ™ immunoassay systems. The principle of the assay is based on chemiluminescent technology. The principle is a one step sandwich technique, where paramagnetic particles coated with goat anti-mouse IgG/anti-ferritin monoclonal antibody (MAB) complexes are combined with goat anti-ferritin alkaline phosphatase conjugate (AFCP), which bind to ferritin molecules in the sample, and the resulting complex in the formation is an insoluble aggregated turbidity, the signal produced is directly proportional to the concentration of ferritin in the sample. The method has been found to correlate extremely well with the reference enzyme immunoassay (EIA) methodology with good precision (CV < 5%), and has an excellent sensitivity of 0.2 ng/mL

All samples, except those from haemochromatosis patients, were labelled with unique barcode identification numbers, centrifuged and the serum decanted from the primary sample tube into a plain tube which was also numbered and marked with the patient's name and date of birth.

An important part of any assay analysis is the standardisation of the methodology employed. Prior to the analysis, the Beckmann Coulter Access™ analyser was calibrated to ensure that every component and result obtained was reliable. Calibration was performed using the six-point calibration procedures provided by the manufacturer. The calibrators were $S_{0,}$ $S_{1,}$ $S_{2,}$ $S_{3,}$ S_4 and $S_{5.}$ The assigned values were 0, 10, 50, 200, 500 and 1500 ng/mL respectively.

Three levels (Level 1, 2 and 3) of commercially prepared controls known as Liquid Assayed Immunoassay controls from Medical Analysis Systems Inc MAS™ were measured on the Beckmann Coulter Access™ analyser on a daily basis both in the morning and afternoon.

Serum samples were analysed on the Beckmann Coulter Access™ analyser platform within 24 h. The analysis was performed using standard ferritin assay reagent kits and performed according to the manufacturer's instructions (Appendix G).

2.2.4 ELISA methods for soluble transferrin receptor measurement

Initially, soluble transferrin receptor (sTfR) measurements were carried out based on the enzyme-linked immunosorbent assay (ELISA) method. ELISA is described as a method whereby antigen or antibody is bound to a solid

surface or a latex particle. The measurement of sTfR was carried out using the reagent kit from Quarantine® IVD® *in-vitro* diagnostic produced by R&D systems. The principle of the assay was based on the microplate sandwich enzyme-linked immunosorbent assay (ELISA), by using two different monoclonal antibodies specific for the quantitative determination of sTfR concentrations in human serum and plasma. This assay was slightly different from that of Briggs *et al*., (2001), where they employed a particle-enhanced immunoturbidimetric assay, which was based on the immunoreactions between sTfR and transferrin specific antibodies (Suominen, *et al*., 1999).

The method used in this study was based on the R&D assay and similar to the approach adopted by Flowers *et al*., (1989). All reagents were prepared as described in the kit insert (Appendix H). The wash buffer concentrate was warmed to room temperature, and mixed gently to ensure dissolution of all crystals. To make up the working buffer solution, 20 mL of the wash buffer concentrate was added into 480 mL of deionised water to 500 mL. Each of the three vials of lyophilised human sTfR in buffered animal serum controls were also reconstituted using 200 µL of deionised water and vortexed, allowed to stand for thirty minutes and vortexed again before use. 96-well polystyrene flat bottomed microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against sTfR were used for all of the ELISA measurements. Aliquots, 100 µL, of the assay diluents were dispensed into each well of separate micro titre plates using a multi-channel pipette, and 20

µL of the calibrators, controls and samples were added to each well. The sampling procedure was performed uninterrupted and was completed within 15 min. The plate frame was then tapped gently to ensure adequate mixing of the contents of each well. The assays were covered with an adhesive strip and were incubated at room temperature (18–25^{o}C) for 1 h. Following incubation, the plates were aspirated and washed four times with wash buffer using the auto washer. Once excess liquid had been removed from the plate, 100 µL of sTfR conjugate was added to each well; plates were then covered and incubated at room temperature (18–25^{o}C) for 1 h. When the incubation time was complete, the washing step was repeated, and 100 µL of substrate was added to each well and incubated at room temperature (18–25^{o}C) for 30 min; direct light was avoided at every stages of incubation. The final step was the addition of 100 µL of stop solution, and the plates were gently tapped to ensure thorough mixing of the contents and the optical density of each well was read within 30 min on a Ceres 900i microplate reader set at 450 nm, according to the manufacturer's instructions. A standard curve was generated and results were automatically calculated by means of the standard curve (Appendix H). All sera were tested in duplicate and the arithmetic mean of any optical density calculated before further analysis was undertaken.

Three controls were provided within the sTfR assay kit and were analysed alongside the patient samples in the same manner as the methodology described above. Expected values provided were as follows:

Table 2.1: sTfR R&D assay: manufacturer control ranges

Control	**Expected range**
Level 1	5.20–7.60 nmol/L
Level 2	12.7–18.5 nmol/L
Level 3	34.6–48.8 nmol/L

The ELISA method described above is used widely in clinical practice and was the method of choice for measuring the sTfR concentration in this study. However, there have been many published reports detailing the inconsistency and lack of standardisation between the methods of sTfR assays, as a result of proteins used in the preparation of standards in different methods. Previous workers claimed there has been possible overestimation of sTfR in patient samples (Brugnara, 2003; Kogan *et al*., 2007).

2.2.5 Zinc protoporphyrin assay using the haematofluorometry principle

Measurements of ZPP were performed using a ZP Haematofluorometer, Model 206D (Aviv Biomedical, Inc). The ZPP Haematofluorometer analyser is

a bench top single channel front surface photofluorometer. The excitation source was a 12 V, 50 W quartz halogen lamp, and the lamp was turned on automatically each time the 'measure' button was pressed, and the light remained throughout the 9 s measurement cycle. The light was filtered by means of an interference filter transmitting at 415 nm, and the excitation light was directed at the drop of blood on the coverslip to enable minimum fluorescence. The light emitted was collected in a vertical direction, which passed through a narrow band interference filter transmitting at 596 nm, and the result was displayed on the 13 mm 7 segment LED readout.

Three AVIV ZP^{TM} red blood cell commercially prepared control vials (low, medium and high) were used throughout the study. These controls represent actual samples drawn from normal, moderately anaemic and severely lead intoxicated individuals. The expected results for each control were quoted on the packaging inserts in µmole ZP/mole Haem (Table 2.2).

Table 2:2: Ranges for internal quality control for ZPP

Control Number	**µmole ZP/mole Haem**
Level 1	83 (+/- 8)
Level 2	144 (+/- 14)
Level 3	295 (+/- 29)

Evidence from some workers suggested that washing of samples for ZPP estimation is a necessary action before the analysis. Studies in the Mannheim Clinic at the University of Heidelberg, Germany, found a list of 60 drugs that caused falsely elevated ZPP readings in whole blood, and some examples include Claforan, Endoxan and Zantic. Therefore, washing the cells of samples before the analysis of ZPP will remove any fluorescent interference that may result in false ZPP elevation (Hastka *et al*., 1992).

The process of ZPP measurement of patient samples involved pipetting 100 µL of EDTA whole blood into a clean labelled polythene tube (13 x 75 mm). Two mL of saline solution was added to the tube using a pipette, and aspirated up and down to ensure a homogenous mixture. The sample was covered using parafilm and centrifuged at 2000 rpm for five minutes. Following this step, the parafilm was removed and the supernatant discarded, leaving only a small volume of saline over the red cells. This was roughly equivalent to the initial sample volume. A clean Pasteur pipette was then used to place approximately 20 µL of sample onto a clean coverslip, already seated on the haematofluorometer slide holder, and the 'MEASURE' button was pressed. The result obtained was recorded for each sample.

2.2.6 Serum iron assay using the colorimetric principle

Serum iron was measured on the Roche automated chemistry analysers (Roche Diagnostics GmbH, Mannheim) using a colorimetric principle. The principle involves the liberation of Fe^{3+} ions from the transferrin complex, and the reduction of Fe^{3+} to Fe^{2+} ions, which was followed by the reaction of Fe^{2+} to give a coloured complex, which was read photometrically. The method was based on the FerroZine method without deproteinisation.

All the reagents and working solutions used were as per the manufacturer's instructions (Appendix D). Reagents (R1) and (R2) were supplied to use. R1 contained citric acid, 200 mmol/L thiourea: 115 mmol/L and detergent, while R2 contains 150 mmol/L sodium ascorbate, 6 mmol/L FerroZine and preservative was also added. All reagents were stored at 2-8^{o}C. Once opened and loaded on the analyser, the stability was for 28 days. The analyser was configured to perform two points calibration using calibrator for automated systems (CFAS) (Roche Diagnostics GmbH, Mannheim), precinorm and precipath controls as supplied by Roche (Roche Diagnostics GmbH, Mannheim). The limits of detection were determined by calculating the value lying 3SDs above that of the lowest standard (standard 1 + 3 SD, repeatability, n = 21).

Serum samples collected for this project were analysed for serum iron according to the methodology already in use in the biochemistry laboratory.

Internal quality control results were checked on the analyser based on the Westgard rule to determine the analytical precision before the patient samples were run.

2.2.7 C-reactive protein (CRP) using the nephelometric principle

C-reactive protein (CRP) was measured on all the patient's samples using a nephelometric method. The nephelometric principle uses the laser light scattering by immune complexes to measure CRP. Nephelometric and turbidimetric assays have been shown to have excellent correlation (Luis *et al*., 2003). The analysis followed the established protocol in the Immunology section of the laboratory. The Behring Nephelometer II (Dade Behring Inc., Deerfield, IL) is an automated analyser for the determination of plasma protein. It measures the agglutination of particles by quantifying scattered light and the detection limit is greater than 0.0175 mg/dL. Serum samples collected for this project were analysed for CRP according to the methodology already in use in the immunology laboratory. IQC results were checked to determine the analytical precision before the patient samples were tested.

2.3 Statistical analysis

Statistical analysis was performed using SPSS version 18. Data collected during the study was recorded in excel spreadsheets and imported to SPSS. Statistical comparisons were performed using independent t-tests and analysis of variance (ANOVA) where applicable.

The main aim of data analysis was to investigate the diagnostic potential of the currently used haematological and biochemical screening procedures for detecting ID. Therefore, data was evaluated using descriptive analysis, normal and skewed distributions of continuous variable were expressed as mean and median (±) standard deviation (SD) respectively. Receiver operating characteristic (ROC) analysis was utilised to illustrate the diagnostic performance of RET-He and RBC-He. Diagnostic plots were used within the SPSS and the cut-offs point for individual parameters were applied in line with the established guidelines. SF < 20 ng/mL was used to classify patients as having ID, while haemoglobin < 11.5 g/dL for female, and < 13.5 g/dL for male was used to classify patients as having IDA. RET-He and RBC-He of 28 pg were also used for the purpose of determining the threshold for upper of lower limits, and this was also in agreement with findings by others (Thomas & Thomas *et al*., 2002; Brugnara, 2003).

Chapter 3 Results

3.0 Introduction

This chapter describes the results of the study of the frontline haematological and biochemical indices of iron deficiency in comparison with the cytometric indices, reticulocyte (RET-He) and erythrocyte haemoglobin (RBC-He) content in iron status.

The main objectives were to demonstrate the use of RET-He and RBC-He as key independent indicators of iron status, and to compare these with the gold standard diagnostic tests, which is SF in most cases, then decide at what stage the gold standard becomes unreliable to the extent of misleading the physicians. The potential use of both indices in the monitoring of haemochromatosis patients was evaluated. The stability and reproducibility of RBC-He and RET-He and MCV will also be reported within this chapter.

3.1 Study Population

The total sample population used in this study was 800, of which eighty-nine (11.1%) samples were obtained from normal individuals. A total of 611 (76.4%) samples were from male and female patients with the following

diagnoses: rheumatoid arthritis, gastroenteritis and renal conditions at the St James's Hospital. One-hundred samples (12.5%) were also received from haemochromatosis patients, who were part of the pilot studies in the Irish National Blood Centre and St James's Hospital, Dublin to determine if the RET-He and RBC-He could contribute to treatment and diagnosis, relative to other markers of iron indices. All samples were received over a period of five months.

Baseline characteristics for the groups of patients used are described below showing the parameters, mean and standard deviation. The normal individuals all had normal red cell indices and are grouped according to gender. The biochemical parameters were found to be normally distributed within the control group. This was used to determine the gender specific intervals for this study.

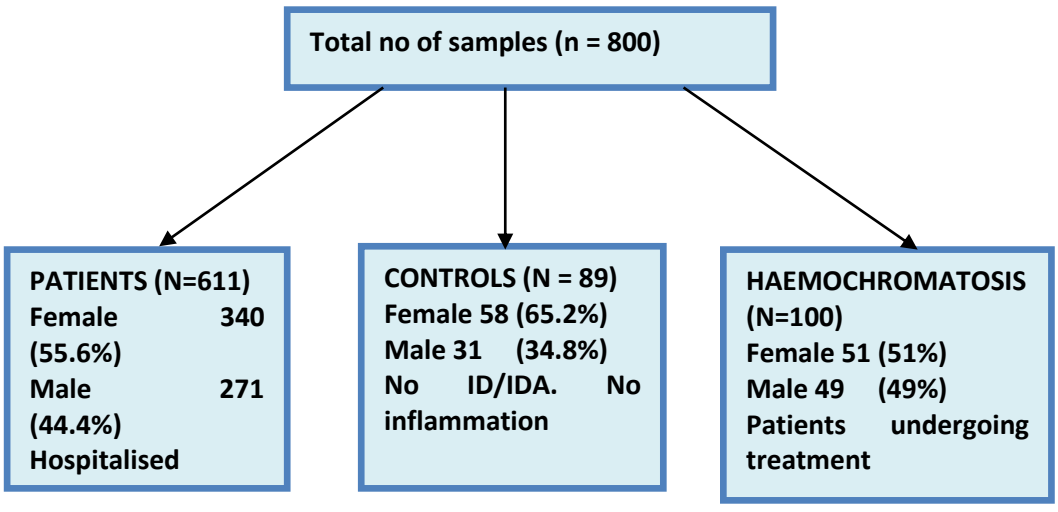


Figure 3:1. Flow chart showing study population

3.2 Stability and reproducibility assessment

Analysis within this study depended on the reproducibility of the measured parameters RET-He and RBC-He. Therefore, the first set of analyses performed was to examine the changes in RET-He and RBC-He (Table 3.1).

Table 3.1: Reproducibility results for the reticulocyte and erythrocyte indices

	RET-He (pg)	**RET-He (pg)**	**RBC-He (pg)**	**RBC-He (pg)**
	SD	CV %	SD	CV (%)
Within-run precision	0.41	1.33	0.12	0.9
SD of the run means	0.38	1.25	0.30	1.0
SD of daily means	0.23	2.94	0.19	0.8
Total Imprecision	0.82	2.85	0.30	1.24

The precision data were collected over 10 days and showed RET-He total imprecision to be SD = 0.82 pg (CV 2.85%). The total imprecision for RBC-He showed an SD = 0.30 pg and CV 1.24% as shown in the table above. Results shows that precision is in agreement with the manufacturer specifications for the parameters measured. All the parameters showed excellent precision, with the coefficient of variation of 2.85% and 1.24% for RET-He and RBC-He respectively. Imprecision obtained for both parameters is therefore negligible. A CV < 5% is an acceptable imprecision for these parameters. Each

run contained internal quality control samples, used as part of the laboratory protocol, and all results were within specified limits of acceptance.

The blood specimens used for the evaluation were residual materials from patient samples sent to the laboratory for routine testing. All samples were drawn into vacutainer K_3EDTA tubes and maintained at room temperature, except during stability studies, in which case samples were stored at controlled temperature as previously described.

3.3 Stability

A total of fifteen samples (normal and anaemic) were selected for the purpose of evaluating the stability of the HB, MCV, MCH, RET-He and RBC-He. Samples (S2, S3, S4, S6, S7, S8 and S15) were considered normal, while samples (S1, S5, S9, S10, S11, S12, S13 and S14) were considered anaemic based on the HB measurements. FBC values were initially measured when samples were received in the laboratory. Aliquots of the selected samples were then stored at 4^{o}C, 20^{0}C or 30^{0}C. All samples were tested at baseline 0, 24, 72 and 96 h to determine if each parameter would change over time. Each run contained internal quality control samples used as part of the laboratory protocol. In the context of sample stability, statistical analysis cannot differentiate between changes due to sample degradation and the

analytical variance due to the limitations of the specified parameters (Imeri *et al.,* 2008).

The figures shown below (Figures 3.2i-3.2v) were obtained from the stability studies performed using the measurement of HB, MCV, MCH, RET-Y and RBC-Y on fifteen samples (S1–S15) obtained from normal and anaemic subjects in different categories. The raw data obtained for the stability studies are presented in Appendix F.

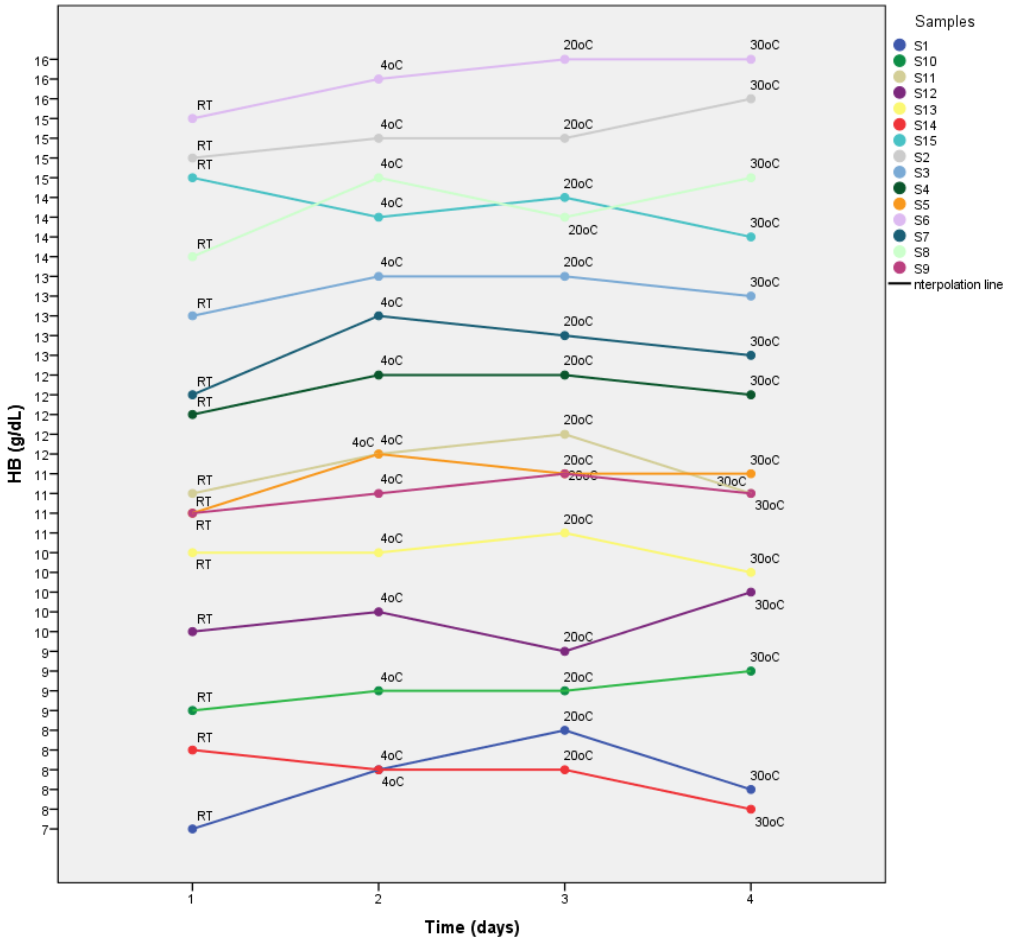


Figure 3.2i: Indicates stability of haemoglobin (g/dL) over 4 days.

HB (g/dL) was considered stable over time at 4^0C, 20^0C or 30 ^{0}C. Changes observed were negligible as shown in Figure 3.2i. Analysis of variance (ANOVA) indicates no significant difference between samples S1 – S15 and between temperatures ($P < 0.05$). The changes observed was not clinically significant and, would not be expected to have serious effect in the identification of patients as either having IDA or not. Delay in sample receipt around this period is not expected to affect the result adversely.

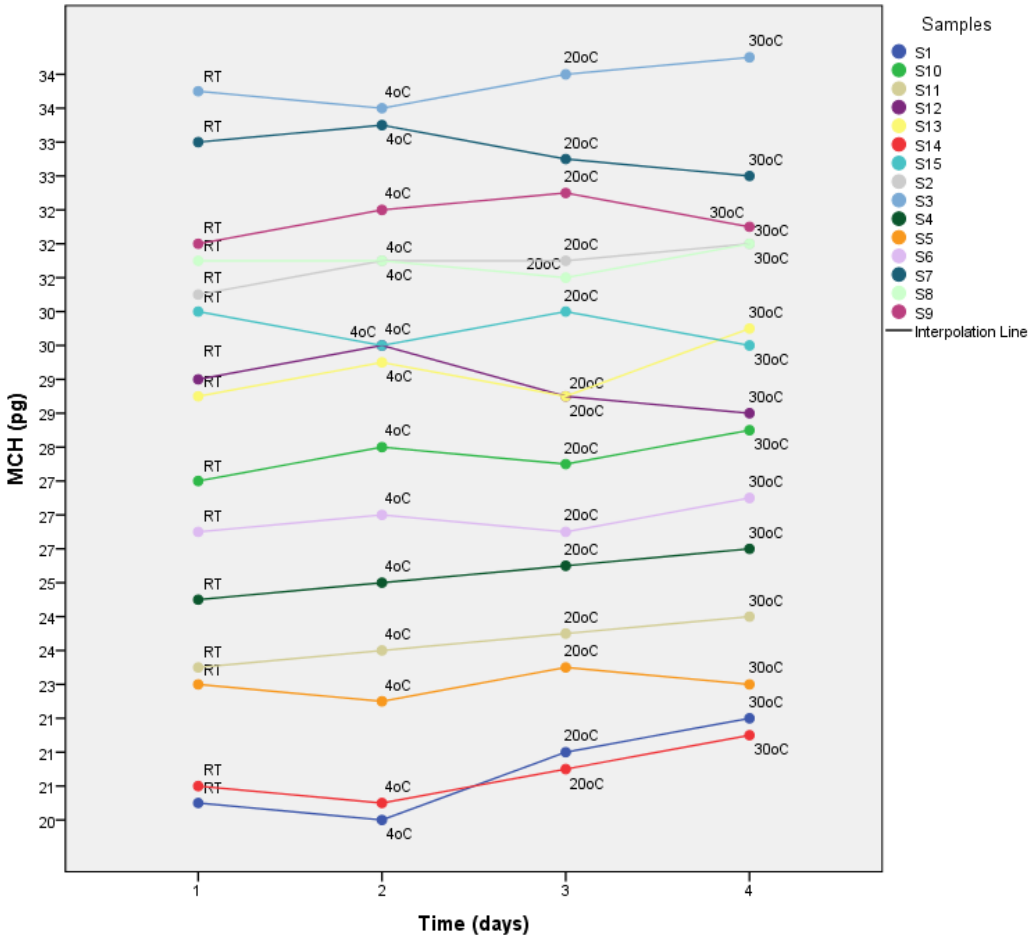


Figure 3.2ii: Indicates stability of mean cell haemoglobin (pg) over 4 days.

MCH was considered relatively stable over time for samples S1 – S15 at 4^0C, 20^0C or 30 ^{0}C. Changes observed were negligible as shown in Figure 3.2ii above. ANOVA indicates no significant changes in MCH (pg) parameter over time with $P < 0.05$. Subsequent analysis indicates results for room temperature (RT) to be slightly lower than results obtained at 4^0C, 20^0C or 30 ^{0}C. The changes observed were not deemed to be clinically significant and would not be expected to affect patient's classification.

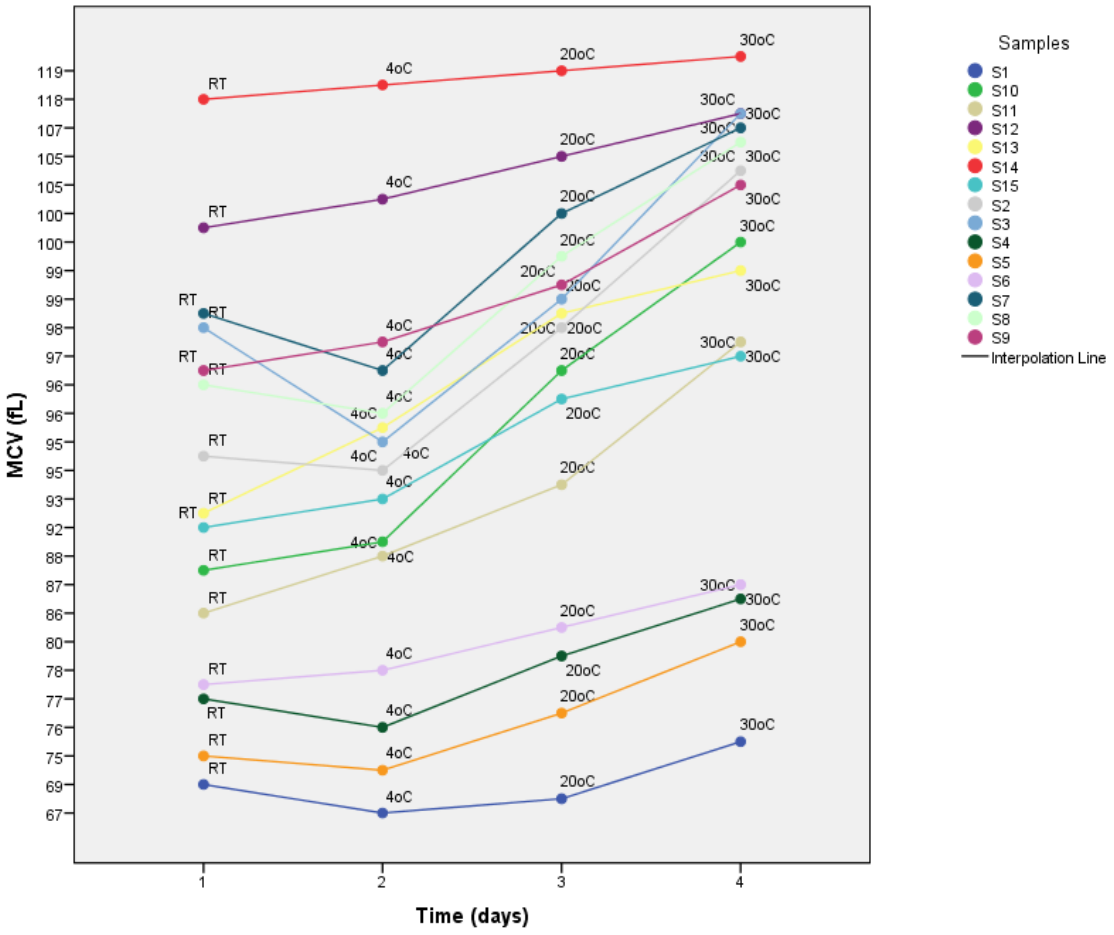


Figure 3.2iii: Indicates stability of mean cell volume (fL) over 4 days

The MCV (fL) was unstable after 24 h, this was due to significant changes in cell size over time at 4^0C, 20^0C or 30 ^{0}C. There were significant changes observed in the MCV (fL) stability for S1 – S15 as shown in Figure 3.2iii. ANOVA indicates significant changes in MCV parameter over time with *P* = 0.06. Repeated analysis indicates the initial results obtained for normal samples (S2, S3, S4, S6, S7, S8 and S15) became higher after 24 h. Also, MCV values in anaemic samples (S1, S5, S9, S10, S11, S12, S13 and S14) became normal after 24 h. The changes observed were considered clinically

significant and, could potentially affect the classification of patients and clinical decision regarding the iron status of an individual.

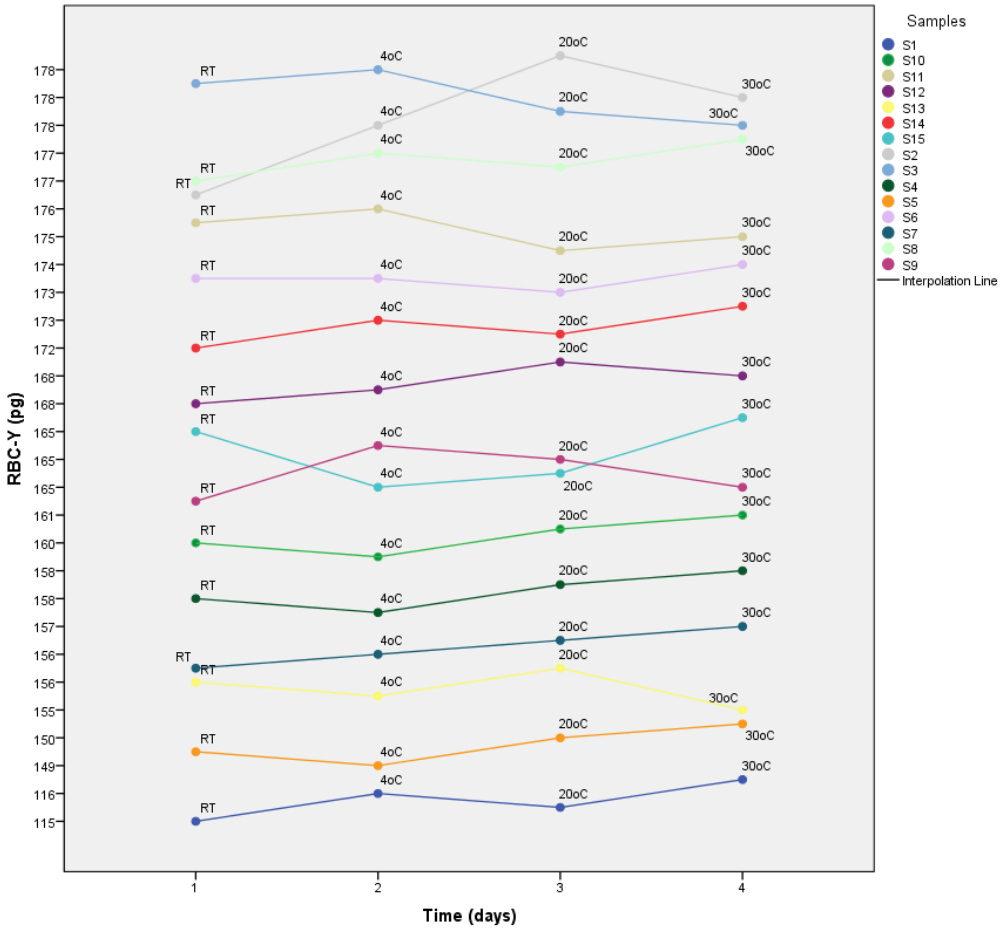


Figure 3.2iv: Indicates stability erythrocyte haemoglobin content (pg) over 4 days.

RBC-Y is considered stable over time for sample S1 – S15 at 4^0C, 20^0C or 30 ^{0}C. There were negligible changes observed in RBC-Y (pg) values as shown in Figure 3.2iv. ANOVA demonstrate there were no significant changes with $P < 0.05$. Repeated analyses indicates marginal difference between results obtained at room temperature with those obtained at 4^0C, 20^0C or 30 ^{0}C. The difference would not have any clinical significant on patient's results.

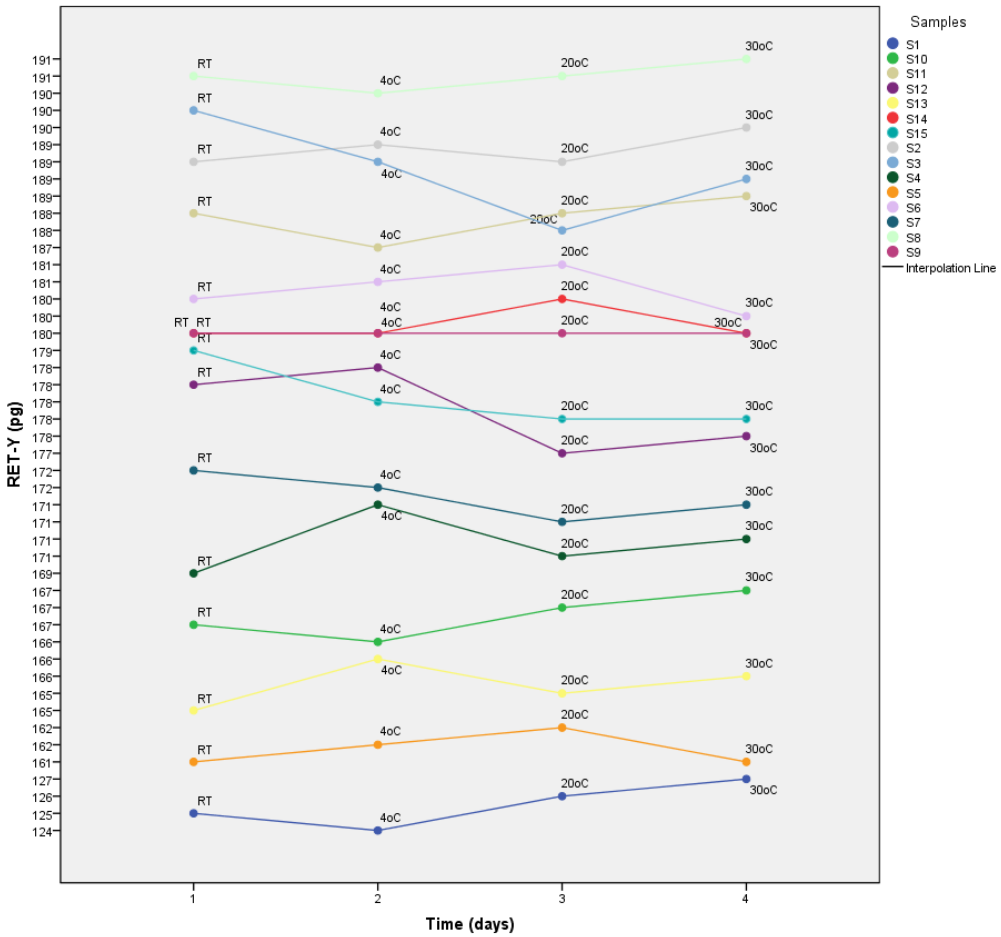


Figure 3.2v: Indicates stability of reticulocyte haemoglobin content (pg) over 4 days.

RET-Y is considered stable over time for samples S1 – S15 at 4^0C, 20^0C or 30 ^{0}C. There were negligible changes observed as shown in Figure 3.2v. Analysis of variance (ANOVA) was used to demonstrate that there was no significant change in RET-Y parameter over time with $P < 0.05$. Repeated analysis indicates results at RT compares with those obtained at 4^0C, 20^0C or 30 ^{0}C. The change observed is not clinically significant.

3.4 Haematological and biochemistry indices in the control group

The normal subjects within this thesis consist of 89 normal adult subjects (58 female, 31 male) without inflammation (CRP < 5 mg/L). The purpose of using these samples was to express the differential results obtained from male and female populations. This also confirmed the understanding gained from many studies, that ID is most common amongst women, especially female of ages 15–49 years old. It is difficult to establish a reference algorithm for the diagnosis of ID for both male and female, even in healthy population as seen in the tables below. The results of male and female should therefore be considered separately (Siti-Noor, *et al*., 2006; Tolentino & Friedman, 2007). Although the numbers considered here may not be sufficient to establish the cut-offs for SF, it does agree with the 5^{th} and 95^{th} percentile values for SF quoted for female (13-130 ng/mL) and male (30–400 ng/mL) (Lotz *et al*., 1997). The ranges quoted by Beckmann Access (male: 24 – 336 ng/mL; female: 11 – 307 ng/mL) are also similar.

3.5 Haematological and biochemical indices in the patients group

The aim of this study was to determine the diagnostic performance of RET-He and RBC-He in comparison with the traditional biochemical and haematological indices of iron deficiency, that is, SF, HB, MCV, MCH, ZPP, and sTfR. Therefore, 611 samples were obtained from patients aged between 18–90 years (271 male; 340 female). Patients were admitted to the following departments at the St James's Hospital, Dublin: Oncology, Urology and Internal Medicine.

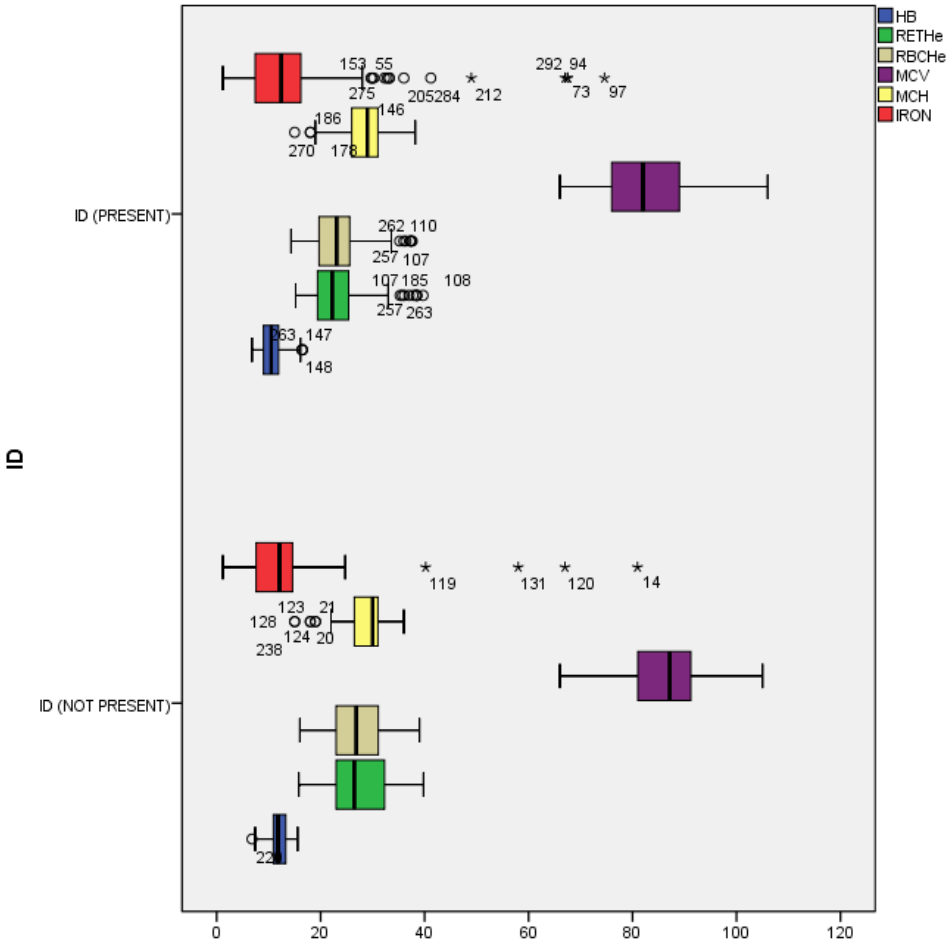
Male and female patients were treated separately. They were classified as either having ID or IDA using SF concentration and haemoglobin levels respectively.

The diagnostic criteria for anaemia in IDA vary between studies. The WHO definition for anaemia is based on age, gender and even pregnancy status. Therefore, some of the criteria set out may not be applicable across every study performed. The HB level < 13.5 g/dL (men) and < 11.5 g/dL (female) were used to define IDA in this study. It is also important to note that pregnant women were excluded from participation in the study. SF ≤ 20 ng/mL was used to classify patients with ID.

Table 3.2: Haematological and biochemical characteristics for 58 normal subjects and 340 female patients.

Parameters	Normal subjects Ranges (Mean)	Iron deficient subjects Ranges (Mean)	Reference Ranges used
Haemoglobin (g/dL)	11.5 – 15.9 (13.7)	6.8 -16.5 (11.3)	11.5 – 16.0
Mean cell volume (fl)	80.1 – 93.4 (88.3)	66.0 - 106.0 (84.1)	78.0 – 101.0
MCH (pg)	25.0 – 35.0 (30.2)	15.0 - 38.2 (28.5)	27.0 – 33.0
MCHC (g/dL)	27.6 – 35.7 (32.1)	21.5 - 35.6 (30.7)	31.0 – 35.0
RDW (%)	11.9 – 18.9 (16.5)	12.0 - 32.0 (16.1)	11.5 – 14.5
RET-He (pg)	27.5 – 34.6 (31.4)	15.2 - 39.8 (24.6)	23.0 – 32.0
RBC-He (pg)	28.3 – 32.5 (30.5)	14.3 - 39.0 (24.8)	23.0 – 32.0
ZPP (µmole zp/mole haem)	58.0 – 91.0 (64.3)	22.0 - 509.0 (90.3)	60.0 – 80.0
Ferritin (ng/mL)	19.4 – 347.1 (65.8)	2.1 - 674.0 (52.0)	12-0 – 200.0
Iron (µmol/L)	4.0 – 58.4 (32.1)	1.2 - 81.0 (13.0)	9.5 – 29.9
sTfR (mg/L)	8.2 – 27.3 (18.4)	2.3 - 102.2 (26.5)	8.7 – 28.0
sTfR Index	7.42 – 22.4 (15.3)	1.7 - 291.1 (25.1)	12.0 – 14.5

Table 3.2 above presents the overall descriptive data obtained for the haematological and biochemical parameters measured in the 340 female samples in this study. Using the criteria described in section 3.5 above, patients were classified into either ID or IDA.

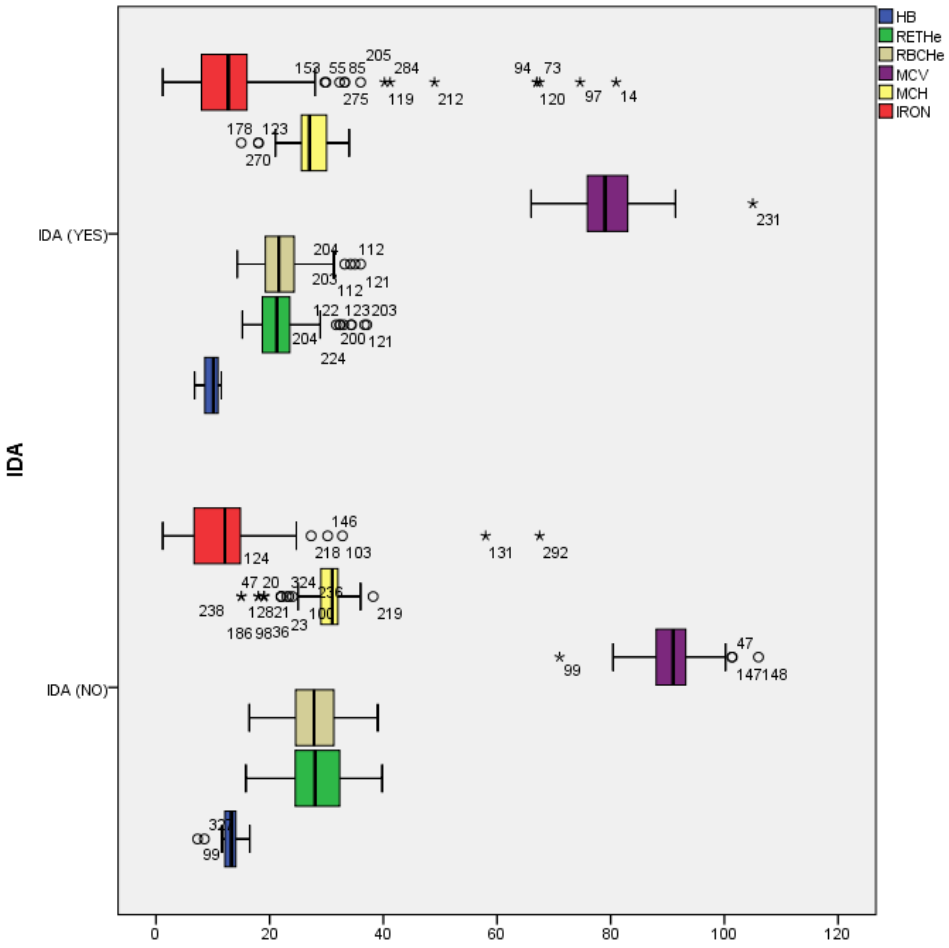


Keywords: Haemoglobin (HB); erythrocyte haemoglobin content (RBCHE); reticulocyte haemoglobin content (RETHE); mean cell volume (MCV); mean cell haemoglobin (MCH); iron; and iron deficiency status (ID).

Figure 3.3: Box plot for female population with ID.

Figure 3.3 above shows the box and whisker plots indicating the preliminary data spread of iron indices distribution versus ID status in the female patient population and also indicates the potential values of individual parameters. The bottom and the top of each box represent lower and upper quartiles (25^{th} and 75^{th} percentiles respectively). The horizontal line inside the box is the median value (50^{th} percentile) for the data distribution for each

parameter presented in the plot. The whiskers at both ends of the box represent the minimum and maximum (5^{th} and 95^{th} percentile) of all data. Data not included are shown as stars and are known as outliers. The data distribution for both RET-HE and RBC-HE seem fairly symmetric as the 'Box' sits in the middle of the range of values for both ID (Present) and ID (Not Present), and the whiskers are approximately equal in length. HB is negatively skewed, and the median is grouped to the left in the negative direction. MCH is also positively skewed. Based on the spread in the Figure 3.3 above, the preliminary display indicates that ID identification using MCV, HB, LOGFERR and MCH may not be as good as the spread for RET-He and RBC-He.



Keywords: Haemoglobin (HB); erythrocyte haemoglobin content (RBCHE); reticulocyte haemoglobin content (RETHE); log ferritin (LOGFERR); mean cell volume (MCV); mean cell haemoglobin (MCH); iron deficiency anaemia status (IDASTATUS).

Figure 3.4: Box plot showing for female population with IDA.

The box and whisker plots as shown in Figure 3.4 above indicates the spread of the preliminary data for the iron parameter in female patient population with IDA. The bottom and the top of each box represent lower and upper quartiles (25th and 75th percentiles respectively). The horizontal line inside the box is the median value (50th percentile) for the data distribution for each parameter presented in the plot. The whiskers at both ends of the box

represent the minimum and maximum (5^{th} and 95^{th}) of all data. Data not included are shown as stars and are known as outliers. The data distribution for both RET-He and RBC-He are fairly symmetrical, as the 'Box' sits in the middle of the range of values for both IDA (Yes) and IDA (No), and the whiskers are approximately equal in length. HB is negatively skewed, and the median is grouped to the left in the negative direction. MCH is also positively skewed. Based on the spread in Figure 3.4, there was no distinct identification of IDA using LOGFERR and MCH. The aggregate in the box plots indicates that RET-He and RBC-He could potentially identify IDA, HB and MCV appear promising.

A receiver-operator characteristics (ROC) curve was constructed with reference to the criteria for classifying patients into either ID or IDA for the 340 female patients.

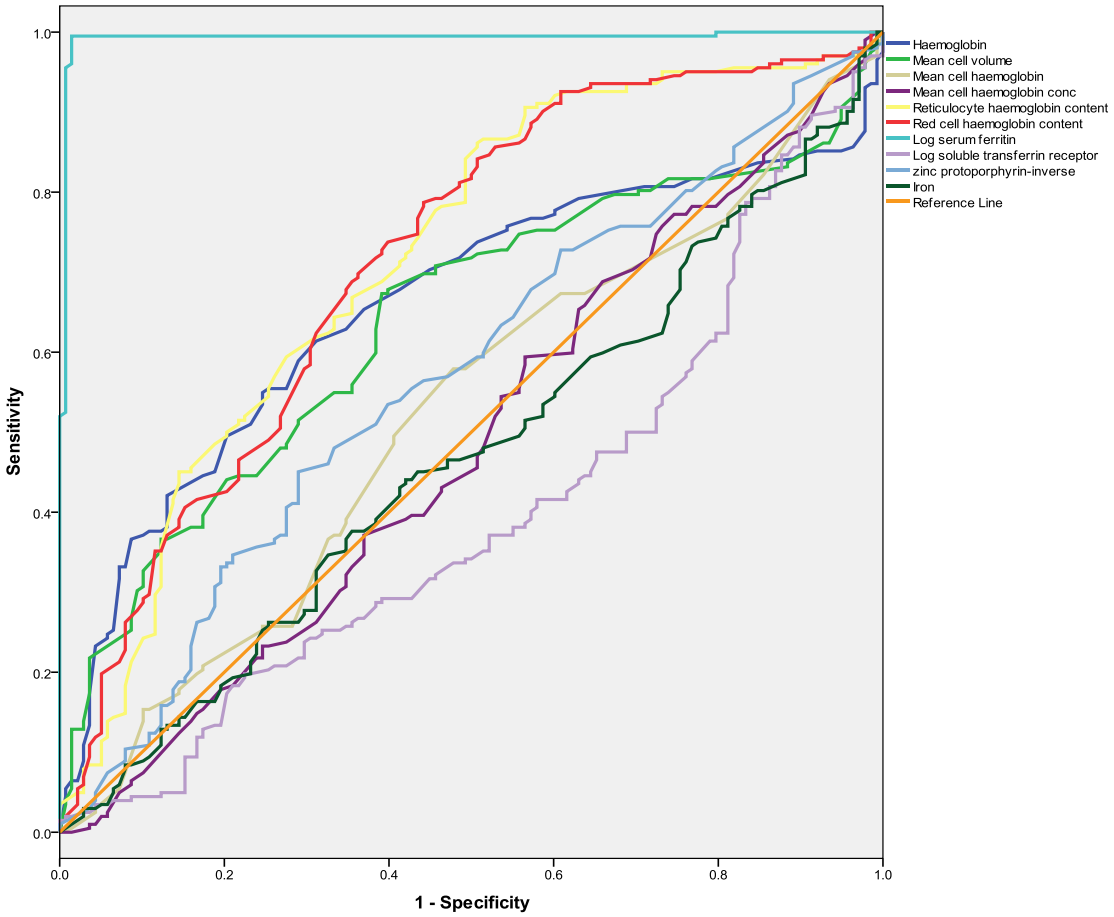


Figure 3.5: ROC for female population with ID classification using SF

Table 3.3: AUC_{ROC}, sensitivity and specificity for iron indicators in female ID

Parameters	**Cut-Off Point**	**AUC_{ROC}**	**Sensitivity %**	**Specificity %**
HB (g/dL)	≤ 11.5	0.66	67.8	59.0
MCV (fl)	≤ 80	0.64	39.6	83.0
RET-HE (pg)	≤ 28	0.72	89.6	58.0
RBC-HE (pg)	≤ 28	0.72	88.6	57.0
ZPP (µmole zp/mole haem)	≤ 70	0.43	59.4	28.0
SF (ng/mL)	≤ 20	0.99	100.0	99.9

Tables 3.3 as shown above presents the sensitivity and specificity of each parameter. SF ≤ 20 ng/mL was chosen to classify patient as having ID. The diagnostic performances of RET-He and RBC-He in ID were compared with the traditional parameters of ID. By using RET-He ≤ 28 pg, ID in female patients within this study could be diagnosed with a sensitivity of 89.6% and a specificity of 58.0%. Also, using a cut-off level RBC-He ≤ 28 pg, ID could be diagnosed with the sensitivity of 88.6% and a specificity of 57.0%. The AUC for SF is 0.99, and SF has 100% sensitivity and specificity. HB has a sensitivity of 67.8% and a specificity of 59.0% ability to identify female patients with ID. In inflammatory condition, the sensitivity and specificity of SF may become unreliable, this is where the real advantage of RET-He and RBC-He would be seen.

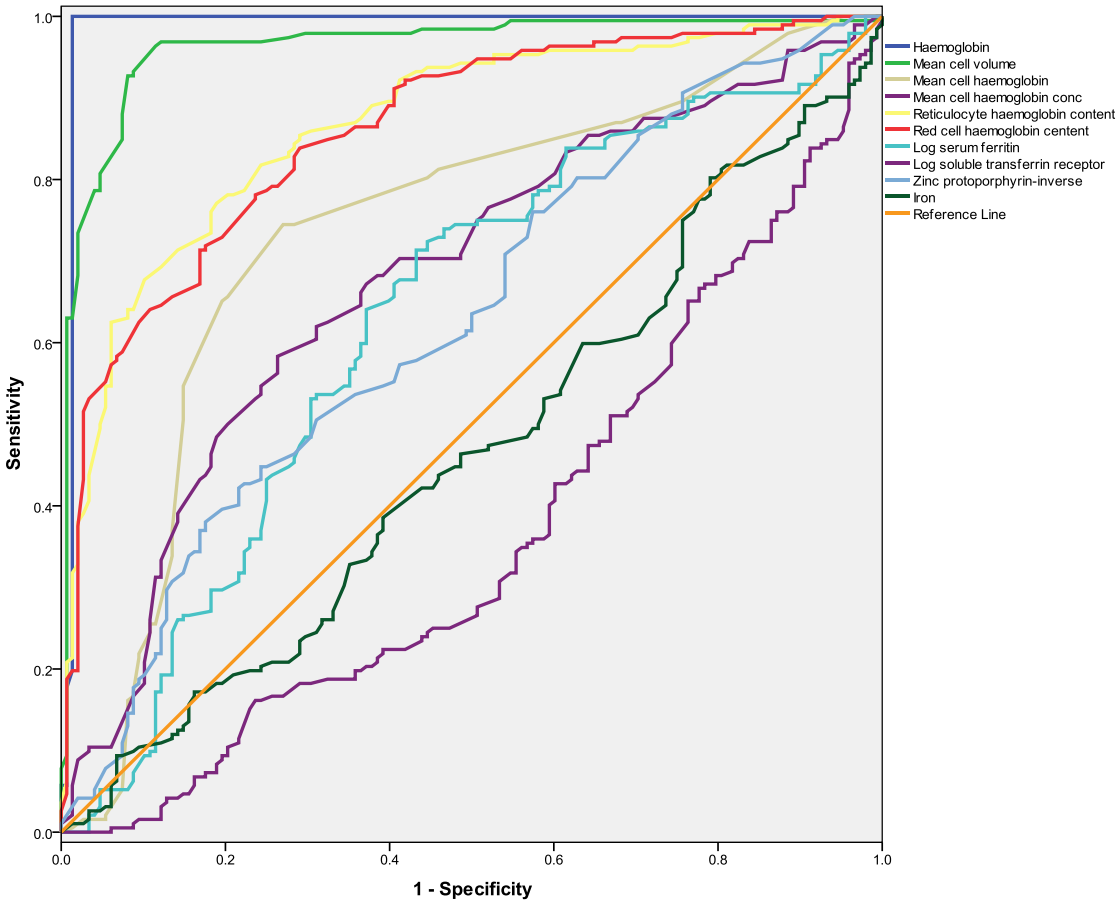


Figure 3.6: ROC for female population with IDA classification using HB

ROC constructed as shown in Figure 3.6 above indicates the performance of each parameter for female patients with IDA classification. A cut-off point of HB ≤ 11.5 g/dL was chosen as the value most suited and close to the highest sensitivity for distinguishing IDA.

Table 3.4: AUC_{ROC}, Sensitivity and specificity for iron indicators in female with IDA

Parameters	**Cut-off Points**	**AUC_{ROC}**	**Sensitivity %**	**Specificity %**
HB (g/dL)	≤ 11.5	0.99	100.0	99.9
MCV (fl)	≤ 80	0.96	61.0	98.0
RET-HE (pg)	≤ 28	0.87	84.0	72.0
RBC-HE(pg)	≤ 28	0.86	82.0	71.0
ZPP (µmole zp/mole haem)	≤ 70	0.37	55.2	24.0
SF (ng/mL)	≤ 20	0.63	71.4	56.0

Tables 3.4 above present the sensitivity and specificity of each parameter. HB < 11.5 g/dL was chosen to classify female patient as having IDA. The diagnostic performances of RET-He and RBC-He in IDA were compared with the traditional parameters of ID. By using RET-He 28 pg, IDA in female patients within this study could be diagnosed with a sensitivity of 84% and a specificity of 72.0%. Also, using cut-off level RBC-He 28 pg, IDA in this group could be diagnosed with the sensitivity of 82.0% and a specificity of 71.0%. The AUC for HB is 0.99, and has a sensitivity of 100% and a specificity of 99.6% ability to identify female patients with IDA.

3.6 Thomas diagnostic plots

The combined results of the iron indices can be displayed in a simple scatter plot known as 'Thomas plot' as coined by its inventors (Thomas & Thomas, 2002). There are four quadrant areas on the plots labelled (A, B, C and D) as shown in Figure 3.7 below. FID can be differentiated from classic ID in hospitalised patients using this diagnostic approach. Quadrant A indicates patients with ACD with full iron stores. The patients within this quadrant are likely to have anaemia, despite normal haemoglobin production. Quadrant B is a transitional area, which shows possible latent iron deficiency. In quadrant C, ACD is more likely presented with full iron stores. This means, we can diagnose FID caused by ACD where reticulocyte cannot appropriately utilise iron for erythropoiesis. Finally, quadrant D would display an uncomplicated and classic ID.

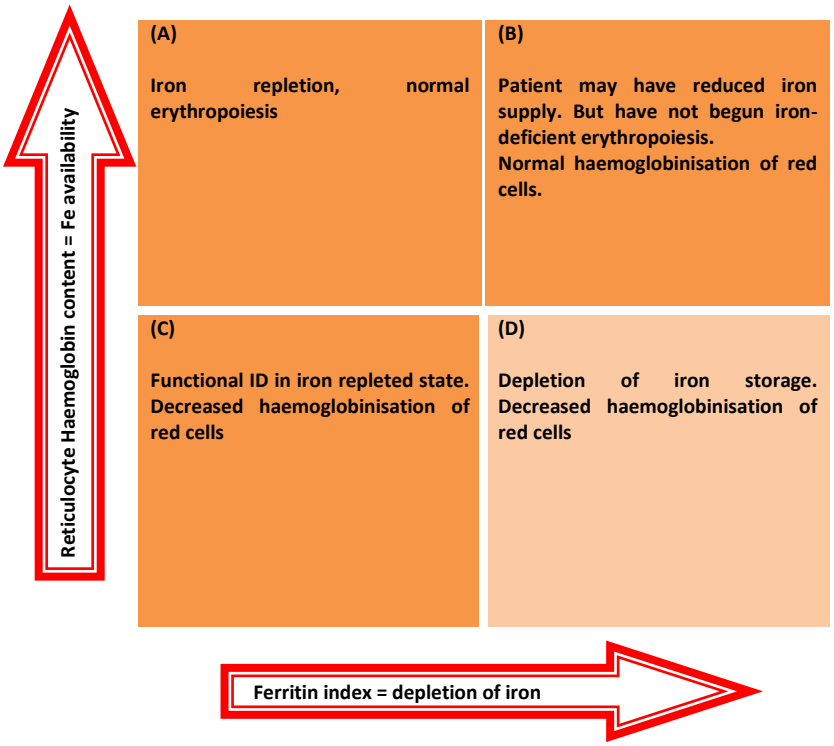


Figure 3.7: Thomas plots showing four quadrants and interpretations.

3.7 Assessment of iron indicators in female patients

To assess the diagnostic relationship between the biochemical and haematological parameters, the following diagnostic plots (Figures 3.8 – 3.19) were used to indicate the ability of a combination of parameters to correctly classify female patients with IDA and ID, even in the presence of inflammation. Parameters were also examined and plotted using the appropriate cut-offs to evaluate individual ability in iron status evaluation.

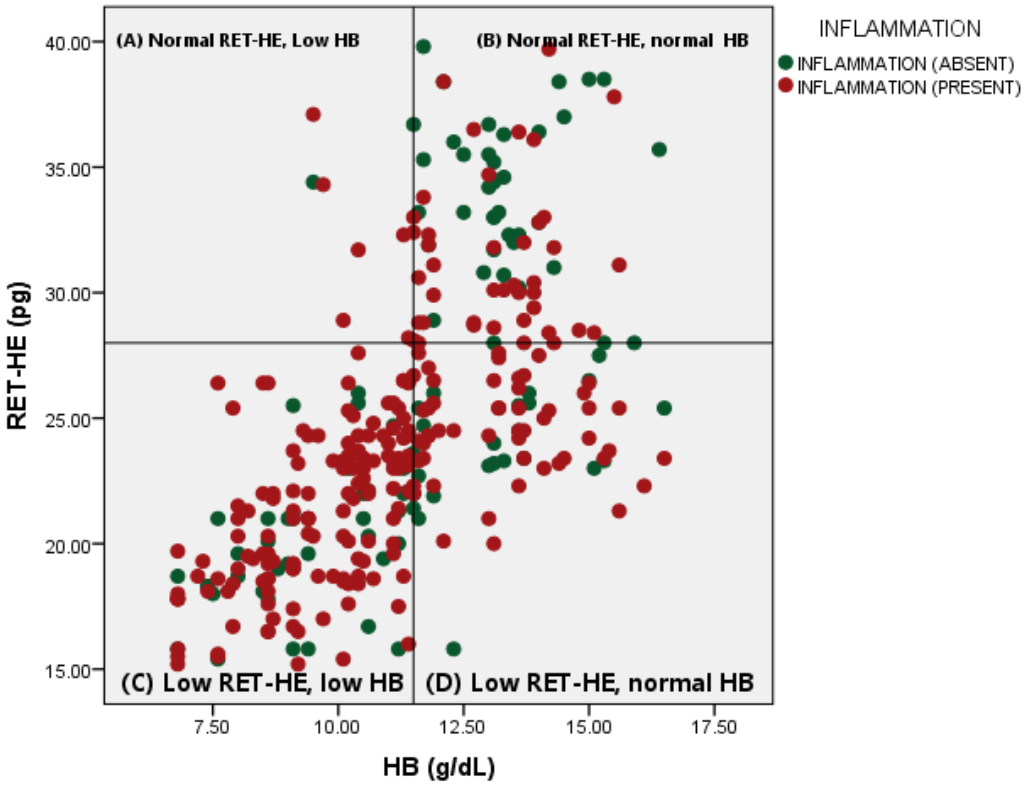


Figure 3.8: Plot for RET-HE versus HB in female (IDA) with or without inflammation

Figure 3.8 above compared RET-He with HB results in female patients with IDA classification. This was to emphasise the role of RET-He in the identification of patients with IDA in the presence of inflammation. Quadrant A represent female patients with normal RET-He and low HB, which is an indication that there was iron repletion despite abnormal haemoglobinisation. These patients are likely suffering from ACD or renal disease and, requiring erythropoietin treatment. Quadrant B represents normal RET-He and normal HB. These patients are part of the population who are not currently suffering from IDA or may have not begun iron deficient

erythropoiesis and, could have been recently treated. Patients within quadrant C were identified as having low RET-He and low HB, the majority of them had inflammation. These categories of patients were considered to have classic IDA and would require treatment. Quadrant D shows patients with low RET-He and normal HB in the presence of inflammation. This is where the real advantage of RET-He is seen. HB here is a late responder and failed to identify these patients. RET-He parameter was able to identify these patients with FID in the presence of inflammation.

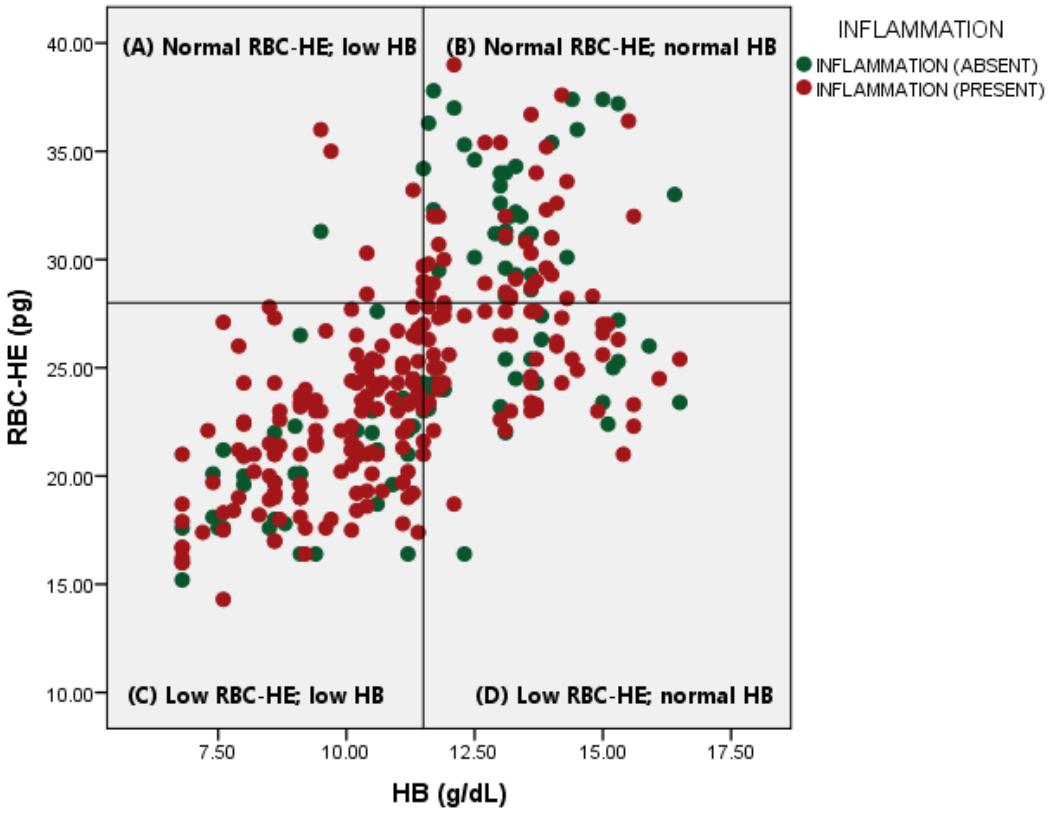


Figure 3.9: Plot for RBC-He (pg) versus HB (g/dL) in female patients with IDA

Figure 3.9 above compared RBC-He with HB results in female patients with IDA in the presence or absence of inflammation. The essence was to see if RBC-He was a better diagnostic indicator to identify female patients with IDA in the presence of inflammation. Quadrant A represents female patients with normal RBC-He and low HB, which is an indication that there was iron repletion despite abnormal haemoglobinisation. Inflammation is also present in all the patients except one. These patients are likely suffering from ACD or renal disease requiring erythropoietin treatment. Quadrant B represents normal RBC-He and normal HB. These patients are part of the population

who are not suffering from IDA or may have not begun iron deficient erythropoiesis. Patients within quadrant C were identified as having low RBC-He and low HB and majority of them had inflammation. The categories of patients were considered to have classic IDA and would require treatment. Quadrant D shows patients with low RBC-He and normal HB in the presence of inflammation. This is where the real advantage of RBC-He is seen. HB here is a late responder to iron deficiency and failed to identify these patients. RBC-He parameter was able to identify the patients with FID in the presence of inflammation.

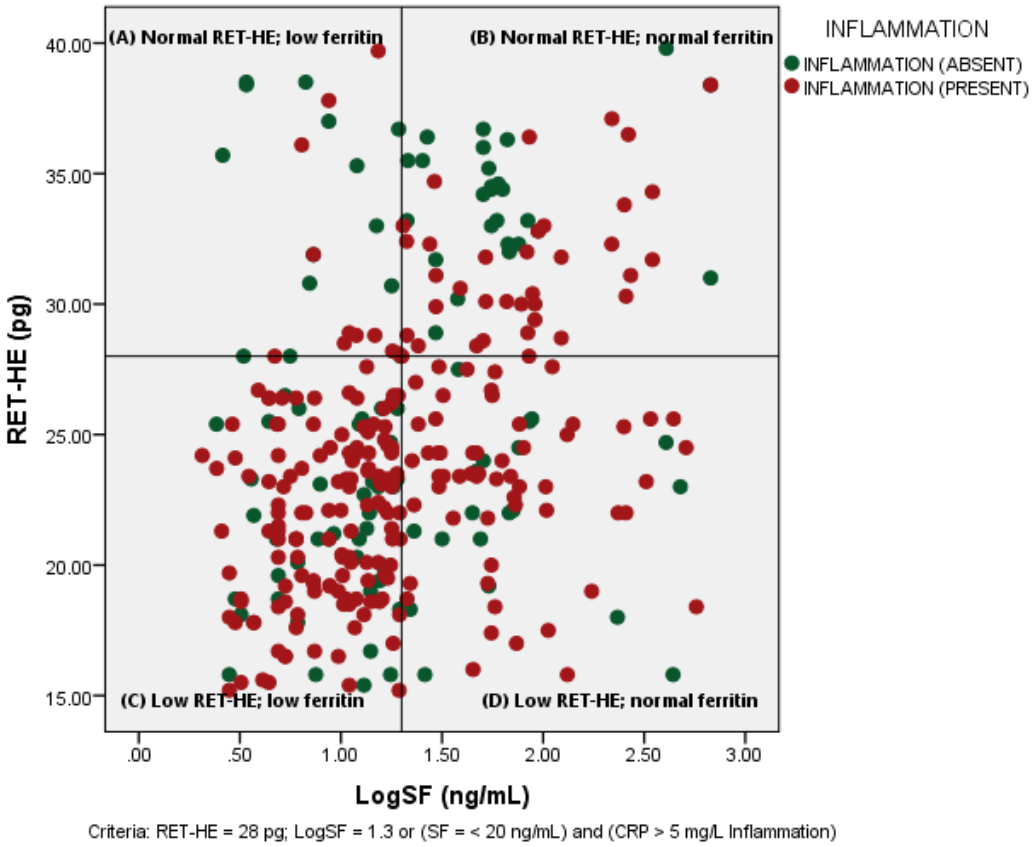


Figure 3.10: Plot for RET-He versus LogSF in female patient with IDA

The diagnostic plot in Figure 3.10 above was used to assess the relationship between RET-He and SF with or without inflammation in female patients with IDA. Quadrant A shows patients with normal RET-He and low SF. Half of the patient population within this quadrant presents with inflammation, and could be classified as having ACD. Quadrant B shows patients with normal SF and normal RET-He, significant number of patients presented with inflammation. These patients are considered as part of the "normal" population who are not suffering from IDA or may have not begun iron deficiency erythropoiesis. Quadrant C represents the patients with low RET-

He and low SF, these patients are most likely suffering from frank deficiency, and have not commenced treatment. Quadrant D shows patients with low RET-He and normal SF. These patients are considered as having classical IDA. The real advantage of RET-He is seen here, and the inflammation may have affected SF.

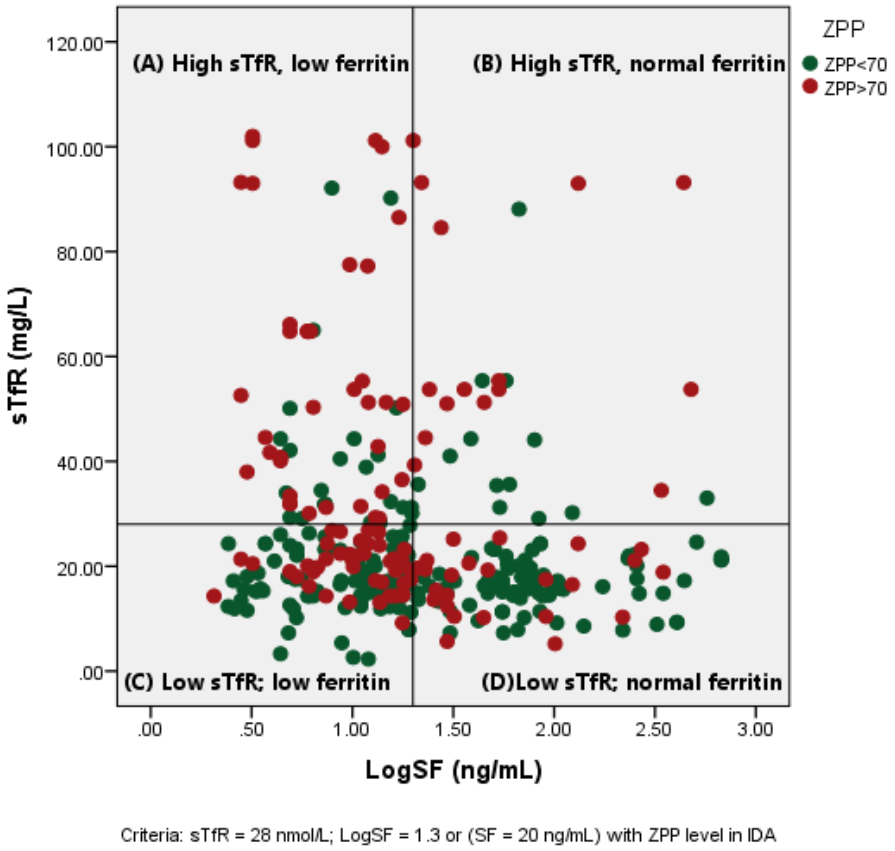


Figure 3.11: Plot for sTfR versus LogSF in female patients with IDA

Figure 3.11 as shown above was used to assess the diagnostic ability between sTfR and SF values in female patients with IDA. Quadrant A shows low SF and high sTfR. The sTfR levels have increased to utilise any available iron within the body. It could be suggested that these patients have IDA or ACD and have not yet started treatment. Quadrant B indicates high sTfR and normal SF, the patients may have been mis-diagnosed as having IDA. Quadrant C patients have low SF and high sTfR. Quadrant D shows normal sTfR and normal SF. This is a clear indication that, the use of sTfR in this group has not particularly provided additional information beyond the ability of SF to reflect low iron.

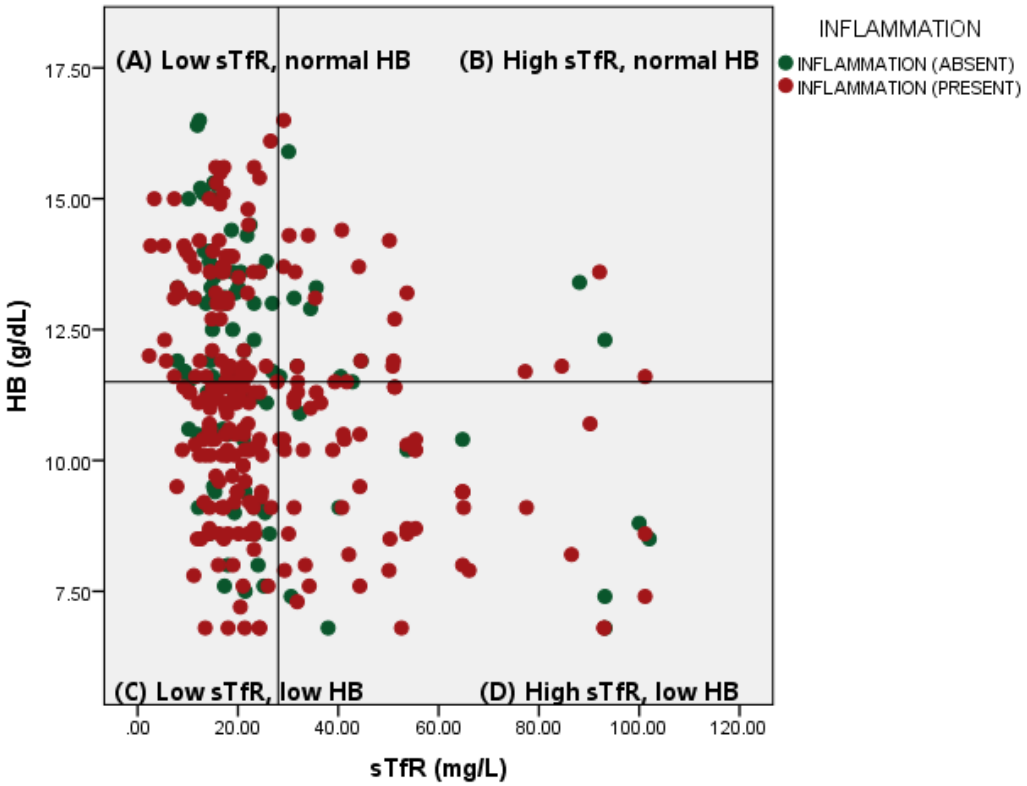


Figure 3.12: Plot for HB versus sTfR in female patients with IDA

Figure 3.12 above compared HB results with sTfR in female patients with IDA classification, and with or without inflammation. The essence was to see if sTfR could provide further information leading to the diagnosis of IDA at the early stage. Quadrant A indicates patients with low sTfR and normal HB with inflammation. Quadrant B shows patients with high sTfR and normal HB. Quadrant C indicating patients with low sTfR and low HB and quadrant D shows high sTfR and low HB. In all the quadrants, sTfR did not indicate female patients with IDA better than the HB has been able to do. It is apparent from the plot that sTfR did not particularly provide additional information in the

patient population, and appears to have not been influenced by inflammation.

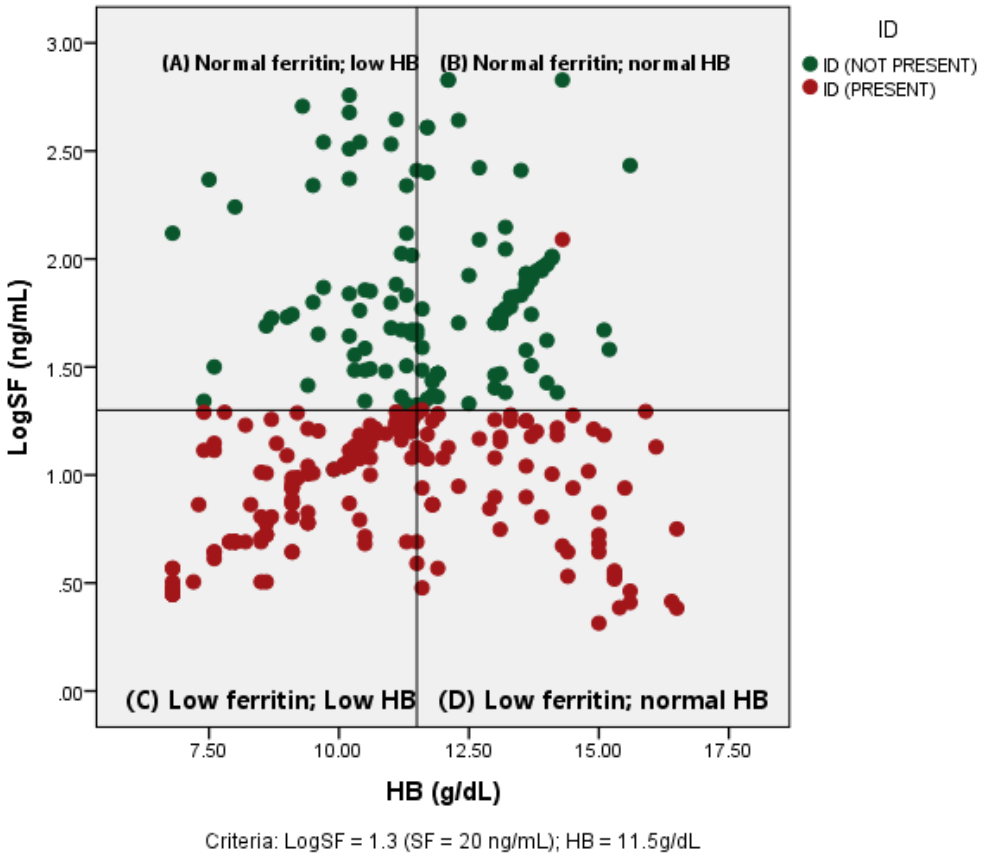


Figure 3.13: Plot for LogSF versus HB in the female patients with ID

The diagnostic plot above was used to assess the relationship between SF and HB in the female patients with ID classification using cut-off point HB ≤ 11.5 g/dL and ferritin ≤ 20 ng/mL. Quadrant A shows normal SF and low HB, this is a likely picture of an ACD. Even in the presence of full iron stores, HB level was low. Quadrant B indicates normal SF and normal HB. These are part of the "normal" population which shows no deficiency or are regarded as latent stage of ID. Quadrant C presents the characteristics of classical ID. It is universally acknowledged that SF is probably the best indicator of ID when it is low. This was indicated in Quadrants C and D. Quadrant D showing normal

HB clearly indicates that HB is a late responder, and would usually be the last indicator to become reduced. If inflammation is introduced, the case may become reversed, and SF could become unreliable.

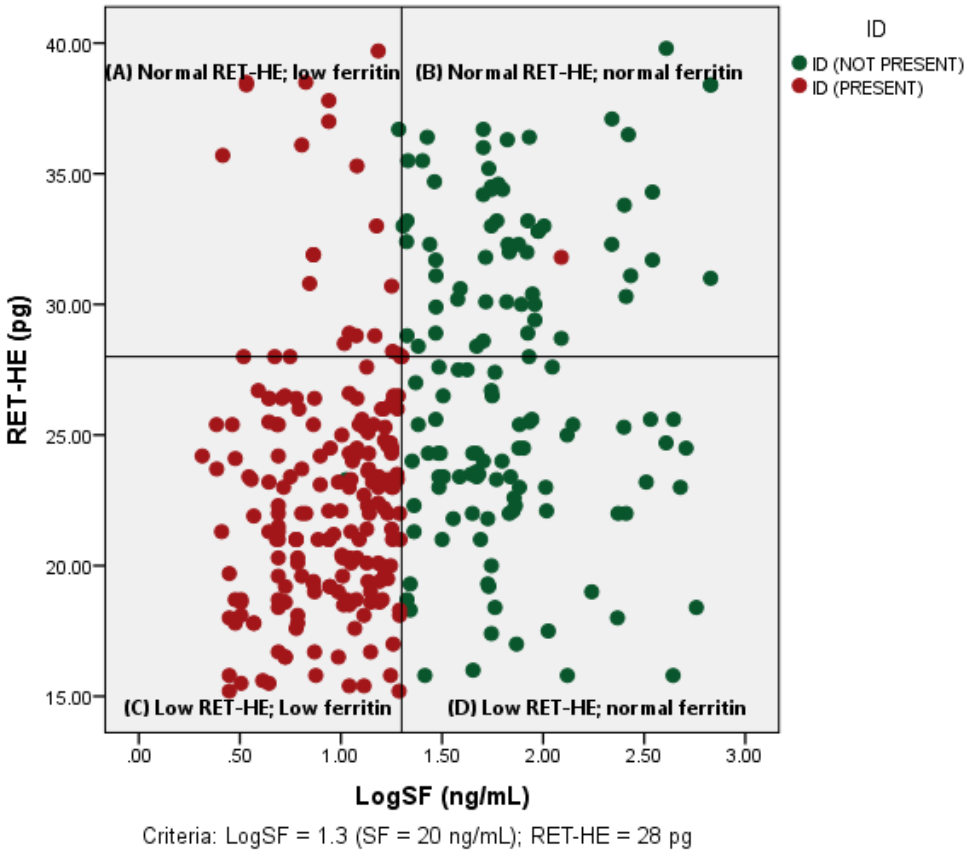


Figure 3.14: Plot for RET-HE versus LogSF in female patients with ID

The diagnostic plot in Figure 3.14 above was used to assess the relationship between RET-He and SF in female patients with ID classification. Quadrant A shows patients with normal RET-He and low SF. These patients are believed to be suffering from ACD and, some of them may be responding to recent treatment. Quadrant B shows patients with normal RET-He and normal SF. These patients are considered as part of the "normal" population who are not suffering from ID or may have not begun iron deficiency erythropoiesis. Quadrant C represents the patients with low RET-He and low SF. These patients are most likely suffering from frank ID, and have not commenced

treatment. Quadrant D represents those with normal iron store and low RET-He. These patients are considered to have classic ID, and this would indicate that SF result could be misleading. The real advantage of RET-He could is seen in this picture.

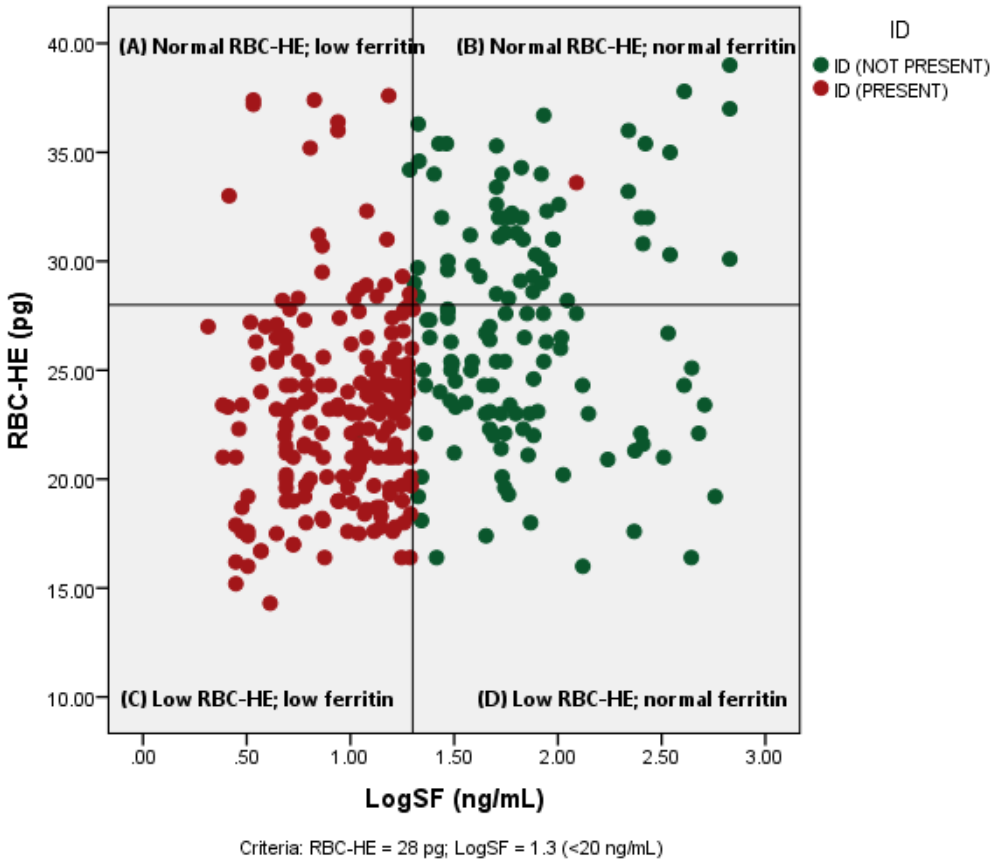


Figure 3.15: Plot for RBC-He versus LogSF in female patients with ID

The diagnostic plot in Figure 3.15 above was used to assess the relationship between RBC-He and SF in female patients with ID. Quadrant A shows patients with normal RBC-He and low SF, patients are believed to be suffering from ACD, and some of them may have been treated. Quadrant B shows patients with normal RBC-He and normal SF. These patients are considered as part of the "normal" population who are not suffering from IDA or may have not begun iron deficiency erythropoiesis. Quadrant C represents patients with low RBC-He and low SF. These patients are most likely suffering from classic ID, and have not commenced treatment. Quadrant D shows patients

with low RBC-He and normal SF. These patients are considered as having classical IDA. The real advantage of RBC-He is seen here.

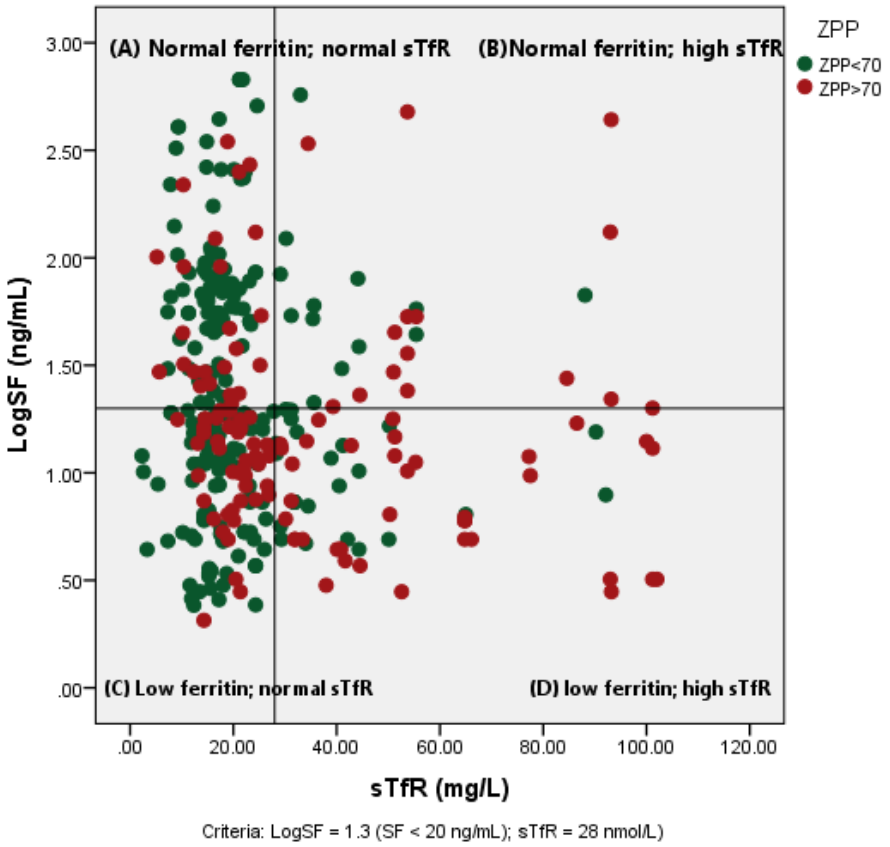


Figure 3.16: Plot for LogSF versus sTfR in female patients with ID

Figure 3.16 as shown above indicates the population of female patients with ID classification. Quadrant A shows normal sTfR and normal SF. Quadrant B indicates normal SF and high sTfR, these patients may have been mis-diagnosed as having ID. Quadrant C shows low SF and normal sTfR, it could be suggested that these patients have ACD and, have not yet started treatment. They are probably still relying on sTfR to improve this deficiency. Also, because of the likely inflammatory states and other conditions as indicated by ZPP > 70 µmolezp/mole haem, the majority of the patients in Quadrant C may have iron that is in the reticulo-endothelial storage and, not being

released for erythropoiesis. The outcome is that the transferrin bound iron, which is reflected by sTfR, becomes low on occasion. Quadrant D patients have low SF and high sTfR. It is interesting to see how many of the patients had elevated ZPP.

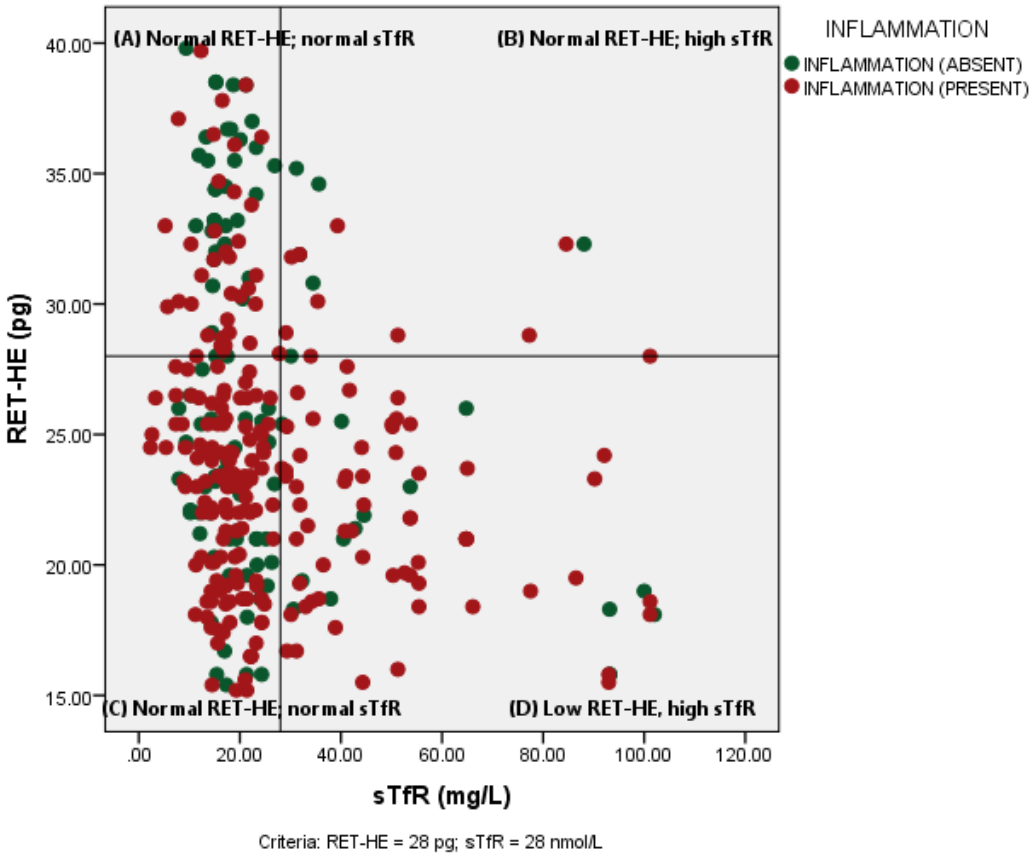


Figure 3.17: Plot for RET-He versus sTfR in female patients with ID

The plot in Figure 3.17 above shows the diagnostic ability of RET-He in comparison to sTfR in female patients with ID. Quadrant A shows normal RET-He and normal sTfR and the majority of the patients had inflammation. Quadrant B indicates normal RET-He and high sTfR. Quadrant C shows low RET-He and normal sTfR, it could be suggested that these patients have IDA or ACD, and have not yet started treatment, as they are still relying on sTfR to improve this deficiency. The majority of the patients in Quadrant C may have iron that is in reticulo-endothelial storage and, are not released for erythropoiesis. The outcome is that, the transferrin bound iron, which is

reflected by sTfR, becomes low on occasion. In quadrant D patients have low RET-He and high sTfR, this indicate classical ID and, patients would require treatment. The measurements of RET-He provide useful information in the diagnosis of FID with inflammation. sTfR does not get affected in inflammation, the majority of patients who had normal haemoglobinisation also presented with inflammation.

Table 3.5: Haematological and biochemical characteristics for 31 normal subjects and 271 male patients.

Parameters	Normal subjects Range (Mean)	Iron deficient Ranges (Mean)	Reference Ranges used
Haemoglobin (g/dL)	14.2 – 13.7 (13.7)	6.80 – 19.6 (12.4)	13.5 – 18.0
Mean cell volume (fl)	83.2 – 100.3 (94.8)	66.0 – 109 .0 (88.1)	76.0 – 100.0
MCH (pg)	27.4 – 35.8 (33.2)	18.0 – 38.0 (29.6)	27.0 – 33.0
MCHC (g/dL)	29.2 – 34.2 (32.1)	20.3 – 37.0 (31.8)	31.0 – 35.0
RDW (%)	12.2 – 18.5 (16.1)	11.8 – 27.0 (15.2)	11.5 – 14.5
RET-He (pg)	27.9 – 38.1 (30.4)	15.0 – 39.9 (26.5)	23.0 – 32.0
RBC-He (pg)	28.2 – 36.5 (31.5)	18.0 – 37.0 (26.7)	23.0 – 32.0
ZPP (µmole zp/mole haem)	48.0 – 122.4 (69.3)	18.0 – 450 (74.4)	60.0 – 80.0
Ferritin (ng/mL)	27.3 – 488.0 (96.7)	2.8 – 1489.3 (85.4)	12.0 – 300
Iron (µmol/L)	21.0 – 72.1 (54.3)	1.3 – 67.4 (12.3)	11.6 – 30.0
sTfR (mg/L)	13.2 – 29.1 (19.5)	2.3 – 102.2 (23.8)	8.7 – 28.0
sTfR Index	6.8 – 19.6 (16.3)	1.8 – 292.0 (18.8)	12.0 – 14.5

Table 3.5 above presents the overall descriptive data obtained for the haematological and biochemical parameters measured in the 31 normal subjects, 271 male patient samples and the corresponding reference ranges used. Using the criteria described in section 3.5 patients were classified into iron deficient, IDA and normal.

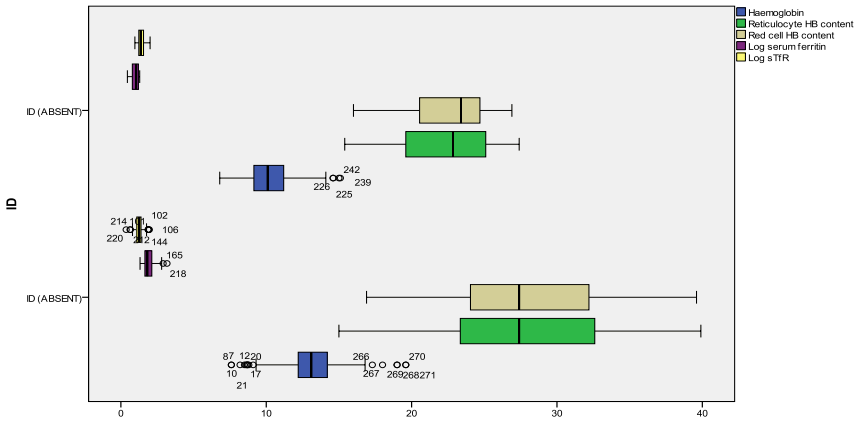


Figure 3.18: Box plot showing the iron indices in male population with ID

The box and whisker plots presented in Figure 3.18 above were used to present the preliminary data spread of the individual iron parameter versus ID status in the male patient population and, to indicate the potential value of individual parameters. The bottom and the top of each box represent lower and upper quartiles (25th and 75th percentiles respectively). The horizontal line inside the box is the median value (50th percentile) for the data distribution for each parameter presented in the plot. The whiskers at both ends of the box represent the minimum and maximum (5th and 95th percentile) of all data. Data not included are shown as stars and are known as outliers. The distribution for both RET-He and RBC-He are fairly symmetrical as the 'Box' sits in the middle of the range of values for both ID (Present) and ID (Not Present). HB was negatively skewed and, the median is grouped to the left in the negative direction. MCH was positively skewed. Based on the spread in the Figure 3.18 above, the preliminary display indicates no distinct identification of ID using MCV, HB, LOGFERR and MCH. The reason could be

as a result of some factors which could have possibly resulted in the overlapping of results. However, the aggregate in the box plots indicates that RET-He and RBC-He could potentially help to identify ID in comparison to the other indices.

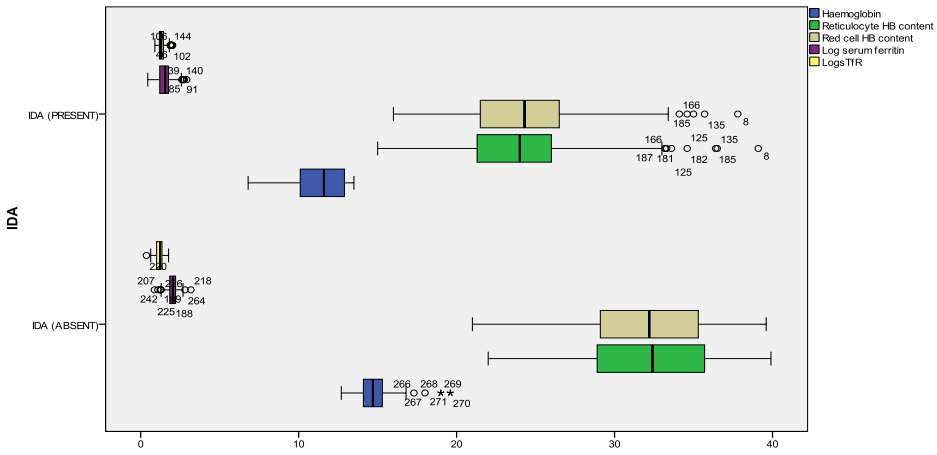


Figure 3.19: Box plot showing the iron indices in male population with IDA

The box and whisker plots presented in Figure 3.19 above were used to present the preliminary data of iron indices distribution versus IDA status in the male patient population and, to indicate the potential value of individual parameters. The bottom and the top of each box represent lower and upper quartiles (25th and 75th percentiles respectively). The horizontal line inside the box is the median value (50th percentile) for the data distribution for individual parameter. The whiskers at both ends of the box represent the minimum and maximum (5th and 95th percentile) of all data. Data not included are shown as stars and are known as outliers. The data distribution

for both RET-He and RBC-He seem fairly symmetric as the 'Box' sits in the middle of the range of values for both IDA (Present) and IDA (Absent), and the whiskers are approximately equal in length. HB is negatively skewed and, the median is grouped to the left in the negative direction. MCH is also positively skewed. The aggregate in the box plots indicates that RET-He and RBC-He could potentially identify IDA. Likewise, HB and MCV appear promising.

A receiver-operator characteristics (ROC) curve was constructed by reference to the set criteria for classifying patients into either ID or IDA for the 271 male patients, and determining the inflammation status of the patients using CRP levels.

The first ROC curve constructed, as shown in Figure 3.20 below was for male with IDA classification. HB ≤ 13.5 g/dL was chosen as the value most suited and close to the highest sensitivity for distinguishing IDA.

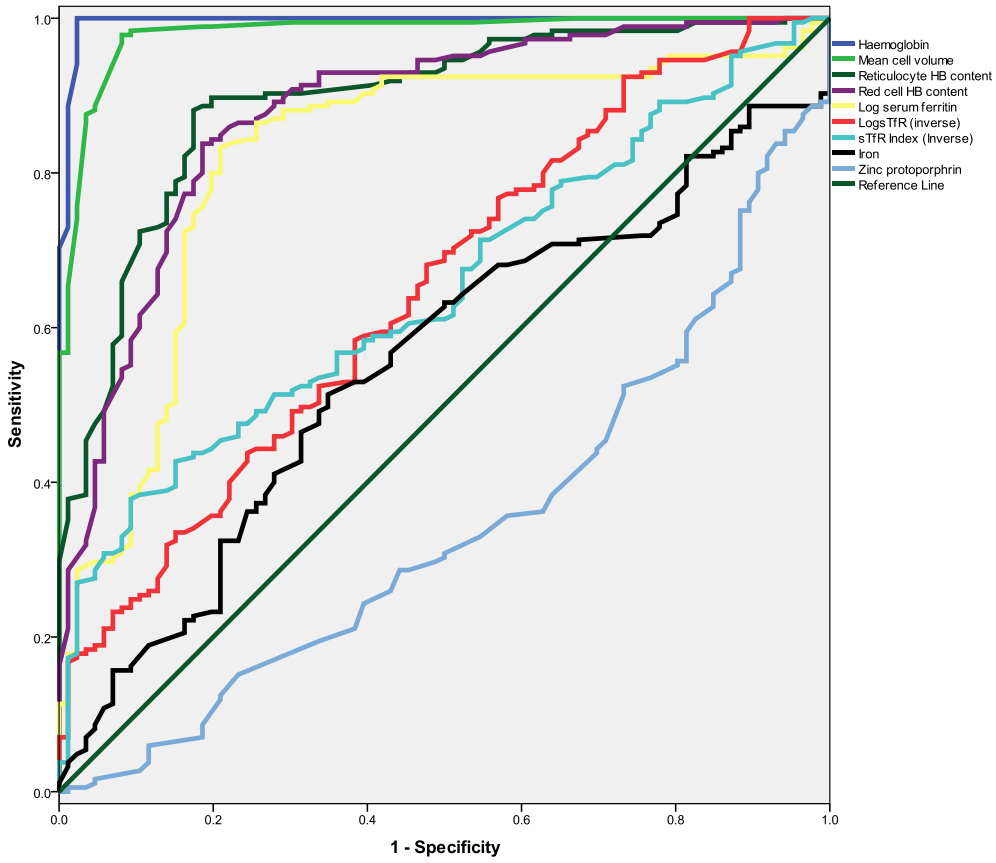


Figure 3.20: ROC curve for the male patients with IDA classification using HB ≤ 13.5.

The ROC curve shown in Figure 3.20 above was for male patients with IDA. A cut-off point of HB ≤ 13.5 g/dL was chosen as the value most suited and close to the highest sensitivity for distinguishing IDA.

Table 3.6: AUC_{ROC}, sensitivity and specificity for iron indices in male with IDA

Parameters	**Cut-off Point**	**AUC_{ROC}**	**Sensitivity %**	**Specificity %**
HB (g/dL)	≤ 13.5	0.99	100.0	99.9
MCV (fl)	< 80	0.98	96.0	94.0
RET-HE (pg)	≤ 28	0.89	88.6	83.0
RBC-HE (pg)	≤ 28	0.87	83.8	80.0
ZPP (µmole zp/mole haem)	≤ 70	0.35	60.0	81.4
SF (ng/mL)	≤ 20	0.82	80.0	64.0

Tables 3.6 above present the sensitivity and specificity of each parameter. HB < 13.5 g/dL was chosen to classify male patient as having IDA. The diagnostic performances of RET-He and RBC-He in IDA were compared with the traditional parameters. By using RET-He < 28 pg, IDA in male patients within this study could be diagnosed with a sensitivity of 88.6% and a specificity of 83.0%. Also, using a cut-off level RBC-He < 28 pg, IDA in male patients group could be diagnosed with a sensitivity of 83.8% and a specificity of 80.0%. The AUC for HB was 0.99 and, has a sensitivity of 100% and a specificity of 99.9% to identify male patients with IDA.

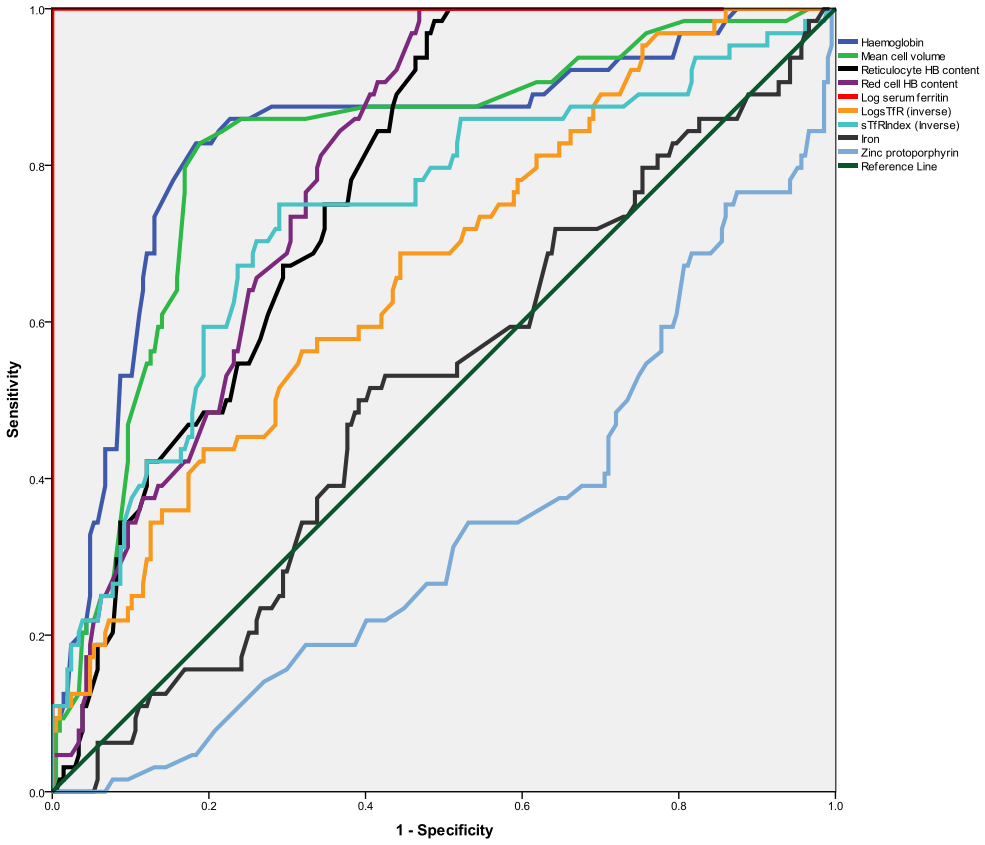


Figure 3.21: ROC for male patients with ID classification with SF≤ 20 ng/mL

The ROC curve constructed, as shown in Figure 3.21 was for male patients with ID. SF ≤ 20 ng/mL was chosen as the cut-off point most suited and close to the highest sensitivity for distinguishing ID.

Table 3.7: AUC_{ROC}, sensitivity and specificity for iron indicators in male with ID

Parameters	**Cut-off Point**	**AUC_{ROC}**	**Sensitivity %**	**Specificity %**
HB (g/dL)	≤ 11.5	0.84	82.8	81.6
MCV (fl)	< 80	0.82	42.2	92.3
RET-HE (pg)	≤ 28	0.78	78.4	66.0
RBC-HE (pg)	≤ 28	0.79	80.0	61.2
ZPP(µmole zp/mole haem)	≤ 70	0.34	48.4	28.0
SF (ng/mL)	≤ 20	1.00	100.0	100.0

Tables 3.7 above present the sensitivity and specificity of each parameter of iron indices. SF ≤ 20 ng/mL was chosen to classify patient as ID. Hence, the strong ability of SF in identifying ID. The diagnostic performances of RET-He and RBC-He in ID were compared with the traditional parameters of ID. By using a cut-off of RET-He ≤ 28 pg, ID in male patients could be diagnosed with a sensitivity of 78.4% and a specificity of 66.0%. Also, using cut-off level RBC-He ≤ 28 pg, ID could be diagnosed with the sensitivity of 80.0% and a specificity of 61.2%. The AUC for SF was 0.99 and, has 100.0% sensitive and specificity. HB has a sensitivity of 82.8% and a specificity of 81.6% ability to identify male patients with ID. In inflammatory condition, the sensitivity and specificity of SF may become unreliable, this is where the real advantage of RET-He and RBC-He would be seen.

3.8 Assessment of iron indicators in male patients

To assess the diagnostic relationship between the biochemical and haematological parameters, the following diagnostic plots were used to indicate the ability of the individual parameters measured to correctly classify 271 male patients with ID and IDA in the presence of inflammation. Parameters were also examined and plotted using the appropriate cut-off values to evaluate individual ability in iron status evaluation.

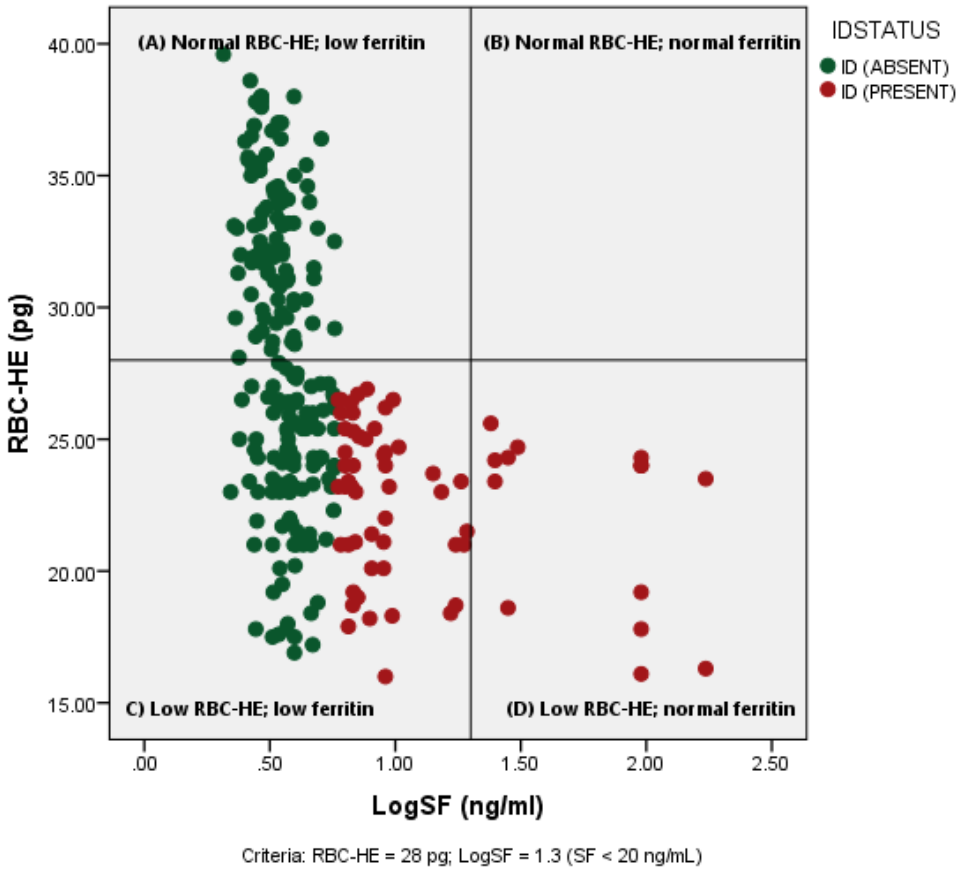


Figure 3.22: Plot for RBC-He versus LogSF in male patients with ID

The diagnostic plot in Figure 3.22 above was used to assess the relationship between RBC-He and SF in male patients with ID. Quadrant A shows patients with normal RBC-He and low SF, patients are believed to be suffering from ACD and some of them may have been treated. Quadrant B shows patients with normal RBC-He and normal SF. These patients are considered as part of the "normal" population who are not suffering from ID or may have not begun iron deficiency erythropoiesis. Quadrant C represents the patients with low RBC-He and low SF. These patients are most likely suffering from classic ID and, have not commenced treatment. Quadrant D shows patients

with low RBC-He and normal SF. These patients are considered as having classic ID. The real advantage of RBC-He is seen here.

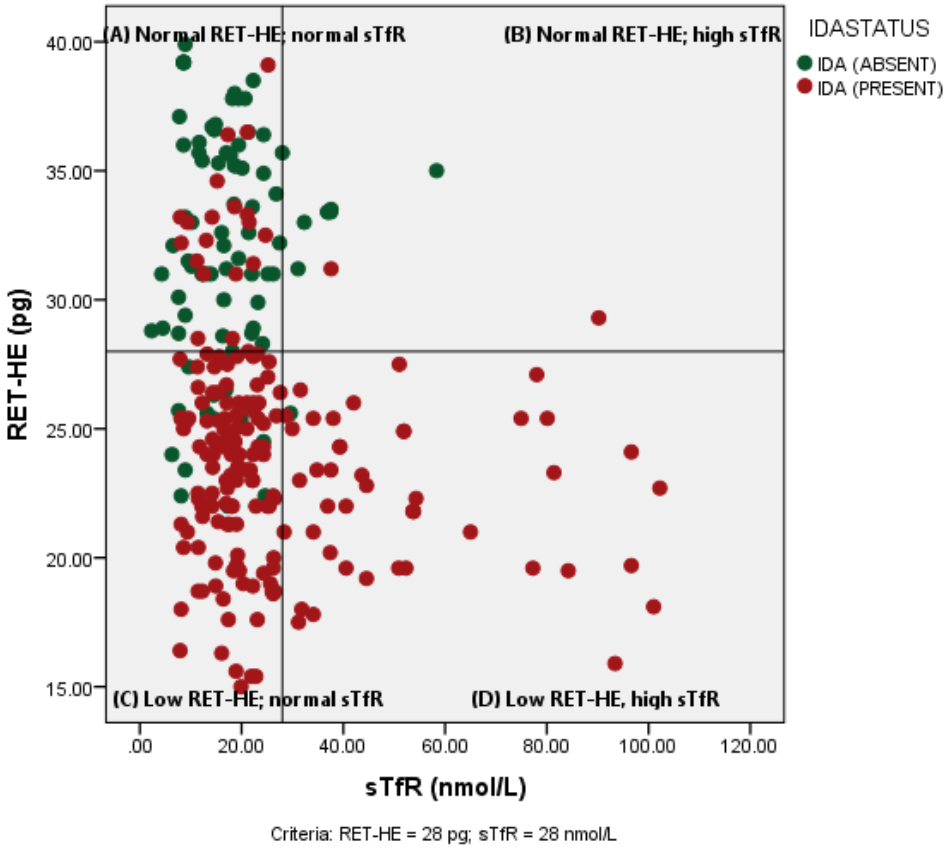


Figure 3.23: Plot for RET-He versus sTfR in male patients with IDA

The plot above (Figure 3.23) shows the diagnostic ability of RET-He in comparison to sTfR. Quadrant A shows normal sTfR and normal RET-He and the majority of the patients had inflammation. Quadrant B indicates high sTfR and normal RET-He. Quadrant C shows low RET-He and normal sTfR, it could be suggested that these patients have IDA or ACD and, have not yet started treatment, as they are still relying on sTfR to improve the deficiency. The majority of patients in Quadrant C may still have iron in the reticulo-endothelial storage, and not being released for erythropoiesis. The outcome is that the transferrin bound iron, which is reflected by sTfR, becomes low on

occasion. In quadrant D patients have low RET-He and high sTfR, which indicate classic ID and the patients, would require treatment. The measurements of RET-He provide useful information in the diagnosis of FID with inflammation. sTfR does not get affected in inflammation, the majority of the patients who had normal haemoglobinisation were also presented with inflammation.

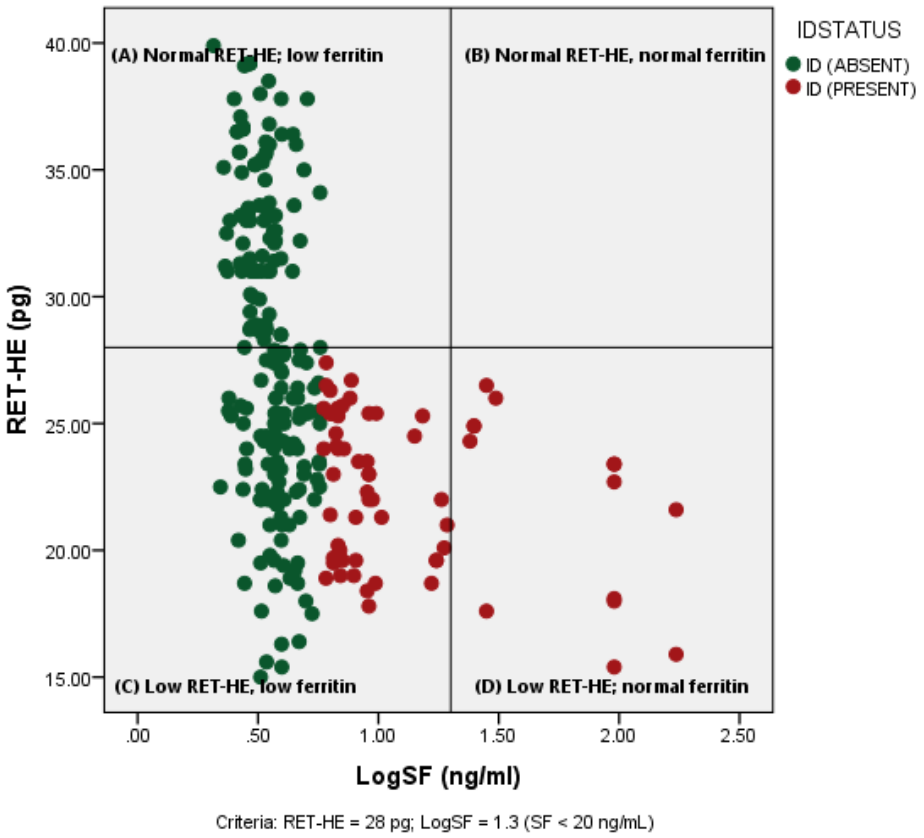


Figure 3.24: Plot for RET-He versus Log SF in male patients with ID

The diagnostic plot in Figure 3.24 above was used to assess the relationship between RET-He and SF in male ID. Quadrant A shows patients with normal RET-He and low SF. Quadrant B shows patients with normal SF and normal RET-He. These patients are considered as part of the "normal" population who are not suffering from ID or may have not begun iron deficiency erythropoiesis. Quadrant C represents the patients with low RET-He and low SF, these patients are most likely suffering from frank iron deficiency and, have not commenced treatment. Quadrant D shows patients with low RET-

He and normal SF. These patients are considered as having classical ID. The real advantage of RET-He is seen here.

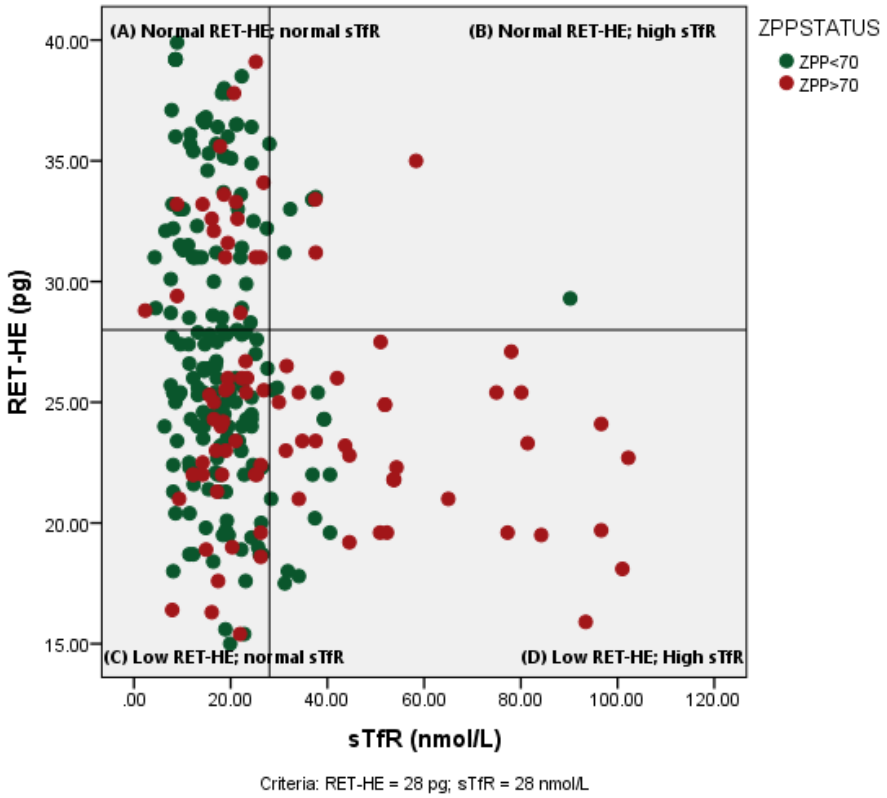


Figure 3.25: Plot for RET-He versus sTfR in male patients with ID

The plot above shows the diagnostic ability of RET-He versus sTfR. Quadrant A shows normal RET-He and normal sTfR, the majority of the patients had low ZPP. Quadrant B indicates normal RET-He and high sTfR. Quadrant C shows low RET-He and normal sTfR, it could be suggested that these patients have IDA or ACD, and have not yet started treatment, as they are still relying on sTfR to improve this deficiency. In quadrant D, patients have low RET-He and high sTfR, which indicate classic ID in this population. The measurements of RET-He provide useful information in the diagnosis of FID. sTfR does not get affected in inflammation, the majority of the patients who had normal haemoglobinisation also have ZPP < 70 µmole zp/mole haem. The usefulness

of the RET-He depends on how good it was at predicting the elevated sTfR values.

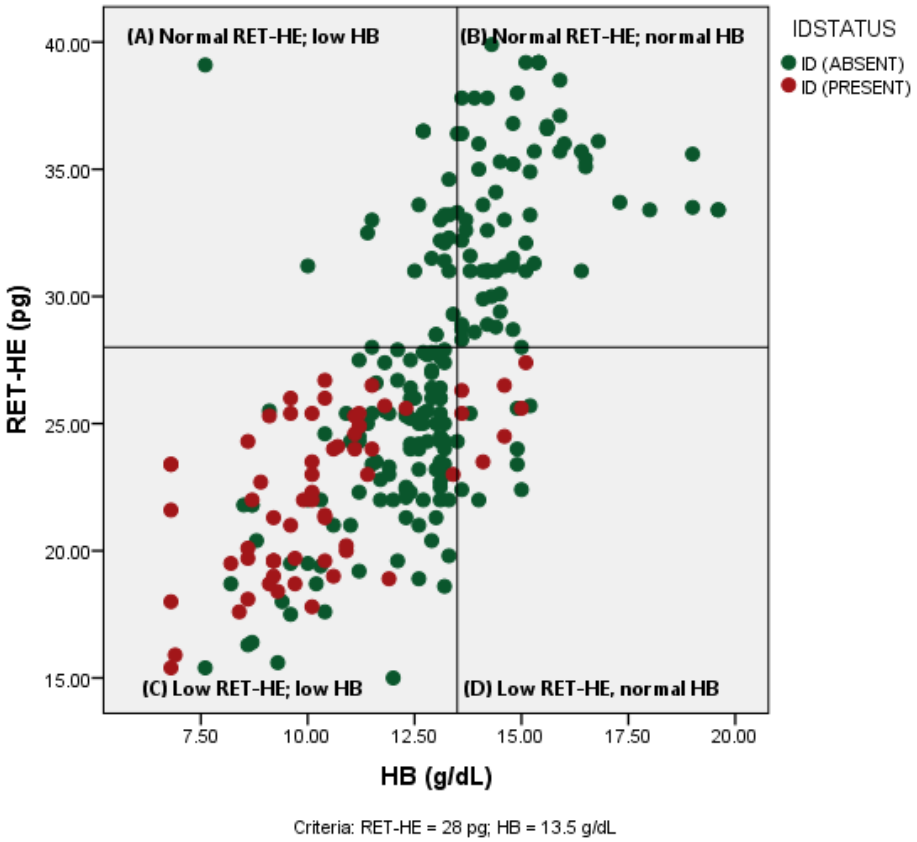


Figure 3.26: Plot for RET-He versus HB in male patient with ID classification

Figure 3.26 above compared RET-He with HB in male patients with ID classification. Quadrant A represent male patients with normal RET-He and low HB, these patients are likely suffering from ACD or renal disease requiring erythropoietin treatment. Quadrant B represent normal RET-HE and normal HB. These patients are part of the population who are not currently suffering from ID or may have not begun iron deficient erythropoiesis or have been recently treated. Patients within quadrant C were identified as having low RET-He and low HB. The categories of patients were considered to have classic ID and would require treatment. Quadrant D shows patients with low

RET-He and normal HB. This is where the real advantage of RET-He is seen. HB here is a late responder to iron deficiency and failed to identify these patients. RET-He parameter was able to identify the patients with FID.

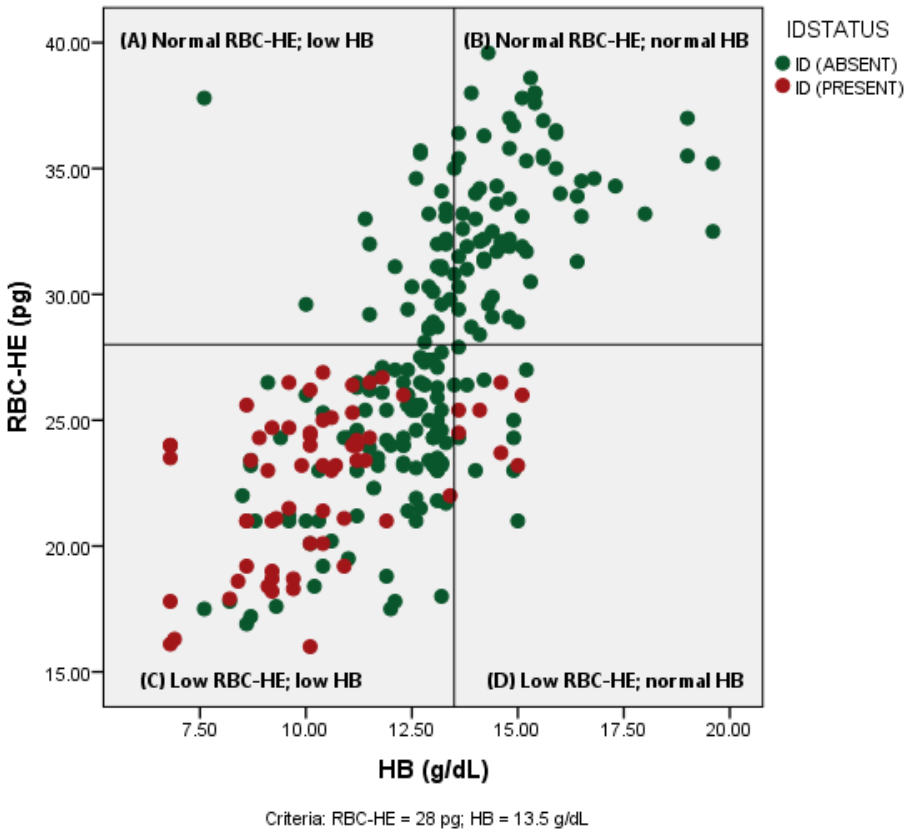


Figure 3.27: Plot for RBC-He versus HB in male patients with ID

Figure 3.27 above compared RBC-He results in male patients with ID classification with HB. Quadrant A represents male patients with normal RBC-He and low HB, which is an indication that, there is iron repletion with decreased HB of red cells. These patients are likely suffering from ACD or renal disease requiring erythropoietin treatment. Quadrant B represents normal RBC-He and normal HB. These patients are part of the population who are not currently suffering from ID or may have not begun iron deficient erythropoiesis or have been recently treated. Patients within quadrant C were identified as having low RBC-He and low HB. The categories of patients were considered to have classic ID and would require treatment. Quadrant D

shows patients with low RBC-He and normal HB. This is where the real advantage of RBC-He is seen. HB here is a late responder to iron deficiency and failed to identify these patients.

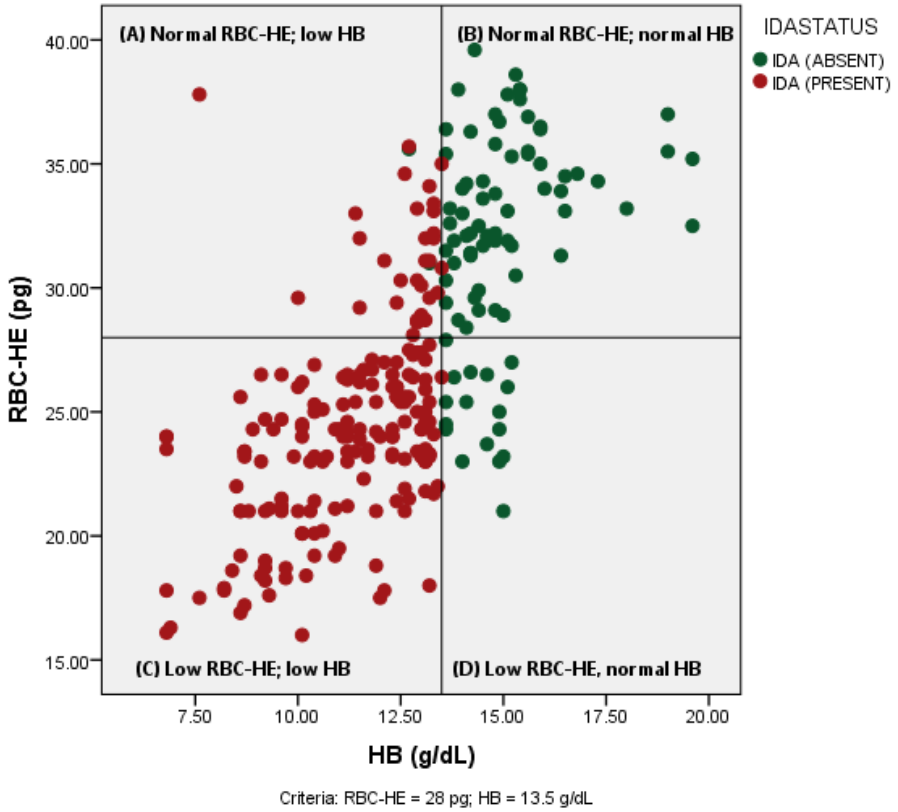


Figure 3.28: Plot for RBC-He versus HB in male patients with IDA classification.

Figure 3.28 above compared RBC-He results in male patients with IDA classification with HB. Quadrant A represents male patients with normal RBC-He and low HB, which is an indication that, there is iron repletion with decreased HB of red cells. These patients are likely suffering from ACD or renal disease requiring erythropoietin treatment. Quadrant B represents normal RBC-He and normal HB. These patients are part of the population who are not currently suffering from IDA or may have not begun iron deficient erythropoiesis or have been recently treated. Quadrant C patients have low RBC-He and low HB. The categories of patients were considered to

have classic IDA and would require treatment. Quadrant D shows patients with low RBC-He and normal HB. This is where the real advantage of RET-He is seen.

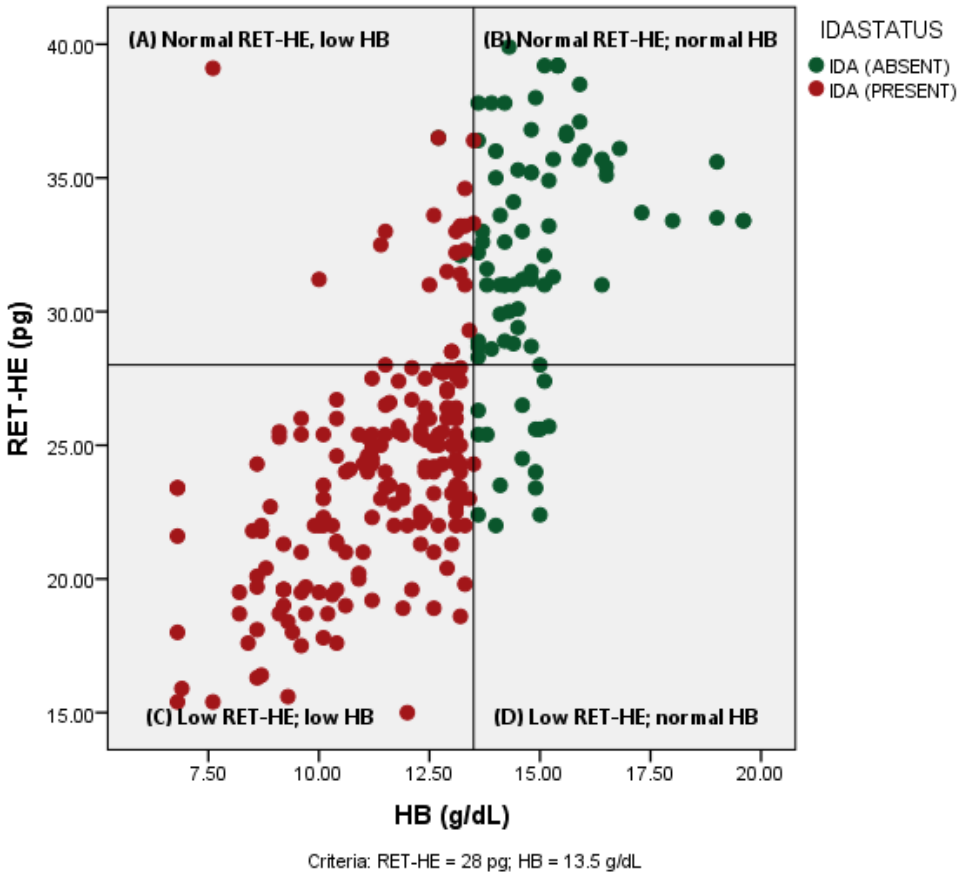


Figure 3.29: Diagnostic plot for RET-He versus HB in male patients with IDA classification.

Figure 3.29 above compared RET-HE results in male patients with IDA classification with HB. Quadrant A represents male patients with normal RET-He and low HB, which is an indication of iron repletion with decreased HB of red cells. These patients are likely suffering from ACD or renal disease requiring erythropoietin treatment. Quadrant B represent normal RET-HE and normal HB. These patients are part of the population who are not currently suffering from IDA or may have not begun iron deficient erythropoiesis or have been recently treated. Patients within quadrant C were identified as having low RET-He and low HB. These patients were considered to have

classic IDA and would require treatment. Quadrant D shows patients with low RET-He and normal HB. This is where the real advantage of RET-He is seen. HB here is a late responder to iron deficiency and failed to identify these patients. RET-He parameter was able to identify the patients with FID.

3.9 Haemochromatosis patients

Traditionally, SF and transferrin saturation have been used to diagnose patients with haemochromatosis. SF correlates well with body iron stores, and an elevated level of SF has been known to have a sensitivity of 80% and specificity of 90% in the diagnosis. Genetic screening has been widely used to define further genotypically the familial occurrence of iron overload associated with C282Y homozygosity or C282Y/H63D compound heterozygosity (Bacon *et al*., 1999). One hundred samples were received from haemochromatosis patients currently undergoing treatment. There was no particular attempt to identify complications as a result of iron overload. However, there were patients with associated conditions like diabetes mellitus and liver cirrhosis. These conditions have been known to be more frequent in C282Y/C282Y than in other pro-bands of haemochromatosis (Sham *et al*., 1997). The aim was to assess the usefulness of RET-He and RBC-He in comparison with HB, HCT and MCV. The RET-He and RBC-He were measured in addition to FBC to determine if both indices could provide the same or improved information when compared with HB and HCT in the patients.

Tables 3.10 and 3.11 below present data obtained from 100 specimens received from haemochromatosis patients during treatment. All participants were white, and the mean age of all persons was 48 ± 15 years (range, 32 –

66 years). 49 (49%) were male, and 51 (51%) were female. There was no significant different in age between men and women.

Table 3.8: Haemochromatosis data - Male patients on treatment

Parameters	**Range Male (n = 49)**	**Agreement with RET-He & RBC-He**
RBC (10^6µL)	3.88-6.35	100%
HB (g/dL)	14.4.0-19.8	99%
HCT (%)	0.39-0.60	99%
MCV (fL)	74.2-108.4	99%
MCH (pg)	24.4-37.4	99%
MCHC (g/dL)	34.6-37.2	100%
RET-He (pg)	25.0-41.6	99%
RBC-He (pg)	22.2-38.3	99%

Table 3.9: Haemochromatosis patients – Female patients on treatment

Parameters	**Range Female (n = 51)**	**Agreement with RET-He & RBC-He**
RBC (10^6µL)	3.24-5.94	100%
HB (g/dL)	13.2-17.5	99%
HCT (%)	0.32-0.55	99%
MCV (fL)	71.2-103.7	99%
MCH (pg)	23.5-35.4	99%
MCHC (g/dL)	33.8-37.0	100%
RET-He (pg)	24.0-40.6	99%
RBC-He (pg)	21.2-37.5	99%

Patients within this category were the subjects of treatment. The mainstay of treatment for haemochromatosis remains phlebotomy. In most cases, a unit of blood (equal to 250 mg of iron), depending on the haematocrit, were removed per week.

The parameters presented in Table 3.8 and 3.9 were measured after the phlebotomy had taken place during one of the treatment visits. Male patients had significantly greater values of RBCs, HB and HCT than female. RBC (male vs female) 3.88-6.35 x 10^6µL vs 3.24-5.94 x 10^6µL; HB (male vs female) 14.4-19.8 g/dL vs 13.2-17.5 g/dL; HCT (male vs female) 0.39-0.60 vs 0.32-0.55%

with P < 0.001 for each comparison. MCV was greater in males than for females (74.2-108.4 fL vs 71.2-103.7 fL with P = 0.02). MCH and MCHC were marginally higher in males than in females (24.5-37.4 pg vs 23.5-36.6 pg; 34.6-37.2 g/dL vs 34.6-37.2 g/dL; P = 0.02) respectively. However, values of RET-He and RBC-He were 99% in agreement when considered alongside HB, HCT and even the MCV values in both males and females (RET-He 25.0-41.6 pg vs 24.0-40.6 pg and 22.2-38.3 pg vs 21.2-37.5 pg). Only one out of 51 samples analysed in the female category, had a reduced RET-He and RBC-He where the HB and HCT were high. The data shown in Tables 3.8 and 3.9 indicates the relative correlation between RET-He, RBC-He and HB, HCT and MCV.

CHAPTER 4 DISCUSSION

In recent years, many of the published articles on ID have discussed areas ranging from screening the healthy individual to diagnosis and prevention. More importantly, many workers have spent time considering the diagnosis of iron restricted erythropoiesis to overload in various stages of disease in different populations. However, the possibility that RET-He and RBC-He indices can be used in the early detection of ID in a larger population of young and adults, in conjunction with other haematological parameters without the need to rely solely on the traditional use of biochemical analysis, has not been fully investigated.

One of the recommendations put forward in the report of a joint World Health Organisation/Centre for Disease Control and Prevention on the assessment of iron status at the population level was that an additional study was needed to validate the recommended indicators of iron status (World Health Organisation, 2004, p94).

Ullrich *et al*., (2005) demonstrated that RET-He should be the preferred screening tool in the early detection of ID and recommended further studies to validate this claim. A review article published by Wish (2006), highlighted the need to weigh the benefits of other tests that may prove useful when

screening for ID especially in challenging cases, rather than the use of the traditional markers (Wish, 2006),

The accurate and early diagnosis of IDA is essential because it might be the first presenting sign of occult bleeding associated with possible malignancies (Willoughby & Laitner, 2000). In the patient group, RET-He provides a snapshot of recent functional availability of iron to the erythrocyte. This group of patients appears to have normal RET-He values, and yet low SF which would indicate that they have recently started treatment for ID. RET-He represents the last 48 h of erythrocyte activity. If the patient is on iron supplements, this would not have been affected. However, the iron stores will take longer to return to normal, so continued iron treatment is necessary to see a return to normal SF values. Intuitively, combining multiple iron status indicators provides the best assessment of iron. Therefore, the combination of HB, MCV, RET-He and RBC-He would provide better diagnosis and guide treatment of ID.

Many studies have also been performed in relation to the use of RET-He and RBC-He, but not in a similar patient population as in this study. The majority of the studies have focussed on patients undergoing chronic haemodialysis, and some other cases where iron therapy was required. Two studies examined the clinical utility of the reticulocyte index (CHr) using the Siemens Advia 120 and 2120, with comparison to other markers of ID. They reported slightly different cut-off values as the optimal CHr cut-off (26.0 and 27.5 pg)

in both studies (Brugnara, *et al*., 1999; Ullrich *et al*., 2005). Also, when the Sysmex XE2100 was introduced to the haematology laboratory, two groups investigated the potential benefits of identifying FID using RET-He. The findings reported that RET-He had a close correlation (in relation) to CHr, sTfR and % hypochromic red cells, as they were known on the ADVIA 120, and the understanding was that RET-He could be an acceptable parameter for indicating FID (Briggs *et al.,* 2001; Thomas & Thomas, 2002). CHr has also been useful in differentiating anaemia and, in monitoring erythropoietin therapy in renal patients undergoing dialysis, the sensitivity was good for detecting relative ID in this study, with a false positive of 20% (specificity 80%) (Briggs *et al.,* 2001; Thomas & Thomas, 2002). The study carried out by Luo *et al*., (2007), compared the diagnostic efficacy of CHr to other markers of ID in Chinese premenopausal women. They recommended further studies to determine whether CHr should be the preferred screening tool in the early detection of ID in larger population of pre-menopausal women and children.

Although studies examining the diagnostic usefulness of various haematological and biochemical parameters have been undertaken, no study of iron status in inflammation has so far included measurements of MCV, SF, MCH, RDW, ZPP, Fe, sTfR, RBC-He and RET-He indices. This study has incorporated all the haematological and biochemical iron indicators and included RET-He and RBC-He as part of the FBC to see if they could provide

additional information on iron status in patients with chronic disease and inflammation.

The results described in this thesis examined the potential value of the currently used haematological screening tests for detecting ID, and compared them with RET-He and RBC-He. Both parameters were found to be useful in the assessment of iron status within the population studied, when considered alongside the traditional markers of ID including SF and sTfR.

It was necessary early in the study to determine the stability of RET-He and RBC-He. This was performed to determine if the parameters would be stable over time, especially when samples arrive late into the laboratory and the analysis is delayed. All the 15 samples were measured for RET-He, RBC-He, HB and MCV, following storage at room temperature, 4^{o}C, 20^{o}C and 30^{o}C over a period of 4 days. Acceptable readings were recorded for all the parameters except MCV. MCV became unstable shortly after 24 h. This effectively limits its diagnostic utility, because results obtained from prolonged or delayed analysis proved MCV values could change dramatically from being very low to becoming normal in patients with ID. This could potentially misguide physicians if they rely on the MCV in the diagnosis of ID. It is useful to know that there were no significant changes in samples analysed when stored over 96 h for RET-He and RBC-He measurement, indicating good stability. Therefore, any delay in sample receipt may not affect the results of RET-He and RBC-He indices.

Reproducibility results for RET-He and RBC-He for within-run and between-run replicate analyses gave an agreement with the manufacturer's specification. The total imprecision was excellent for RET-He (SD 0.82 pg, CV 2.85%); and for RBC-He (SD 0.30, CV 1.24%).

The understanding that iron status can be evaluated by the measurement of iron stores and circulating iron has been known for many years. However, SF and HB may not provide the conclusive answers due to their variability and insensitivity, especially in the presence of inflammation. The imbalance between iron needed by the marrow and the iron supply leads to a reduction of red cell haemoglobinisation. The outcome is hypochromic and microcytic anaemia, which is seen in FID. However, because SF may not accurately reflect FID in situations like this, the determination of the RET-He and RBC-He have been shown to provide useful information in diagnosing FID, and have indeed been offered as a possible alternative to the biochemical markers like iron, SF, transferrin and even sTfR (Brugnara *et al*., 1999; Cullen *et al*., 1999*;* Fishbane *et al*., 1997; Thomas & Thomas, 2002). Also, many of these studies have indicated that biochemical markers of ID are of limited value when assessing ID and FID, and they recommend further studies to assess RET-He and to use different cut-off values. One such study defined FID as a CHr < 28 pg, which was based on the distribution obtained within the healthy population studied (Thomas & Thomas, 2002). Another study indicated that the CHr index will achieve 100% sensitivity when a cut-off is set at 32 pg for

ID (Mitsuiki *et al.,* 2003). Others have suggested that the combined use of sTfR and SF provide better information in chronic inflammation (Benguin, 2003; Bultink *et al*., 2001).

In line with the limitations of the widely used iron status indicators, hepcidin, which is a key regulatory protein in iron metabolism and which acts by down-regulating the release of iron from the duodenum and macrophage, could be a useful marker of iron status (Nemeth, 2004). Reduced serum hepcidin may enable an assessment of iron requirements and provide information regarding the physiological iron deficiency (Kemna *et al*., 2008). Recently, the quantitation of serum and urine hepcidin have been developed using mass spectrophotometry and immunoassay (Koliaraki *et al*., 2009). The clinical utility of hepcidin has been associated with the diagnosis of ACD, chronic kidney disease and haemochromatosis (Koliaraki *et al*., 2009). However, how much information it would add to the existing repertoire of iron is yet to be understood. Various studies have shown a good correlation between serum hepcidin and ferritin, indicating elevated levels for both assays in inflammatory disease (Pidgeon, *et al.*, 2001). However, serum hepcidin has an advantage over ferritin, because of its ability to directly indicate the available need of iron in erythropoiesis. The role of serum hepcidin in relation to RET-He and RBC-He in the assessment of iron status is still not fully understood and would require further investigation. The main shortcoming of serum hepcidin when benchmarked against RET-He and RBC-He is that the

assay has not been fully validated. Some workers found hepcidin level to correlate with CRP, as a marker of inflammation (Peter *et al.*, 2010; Ford *et al.,* 2010). Recently, hepcidin levels were found to predict erythropoietic response to an intravenous iron in haemodialysis patients (Tessitore *et al*., 2010).However, the serum hepcidin assay is not routinely available for use in most laboratories, while RET-He and RBC-He are available on most haematology analysers and, if utilised appropriately, their values would contribute to the assessment of iron status.

In response to the literature, this study was set up to assess and explore the views put forward by these workers, to determine if RET-He and RBC-He as key independent indicators could identify patients with IDA without the need for biochemical analysis. The results from this study showed some measurable outcome for RET-He and RBC-He parameters in patients with ID and IDA. The number of investigations carried out for the screening and diagnosis of patients with or without ID can be minimized, and focus given to the haematological investigations of the red cell and erythrocyte haemoglobinisation assessment.

The criteria used for patient classification as having ID were based on the SF ≤ 20 ng/mL, while HB ≤ 11.5 g/dL for female and ≤ 13.5 g/dL for male was used to indicate patients with IDA. These are in agreement with the published data for SF and sTfR (Vernet & Doyen, 2000; Mast *et al*., 1998). The cut-off value used for both RET-He and RBC-He was ≥ 28 pg. This value is in agreement

with the study conducted with the published data for RET-He (Thomas & Thomas, 2002).

The first report of this study considered the analysis of 89 healthy individuals without inflammation showed that RET-He and RBC-He values ≥ 28 pg would indicate iron sufficiency. This is in agreement with other published data which quoted CHr < 28 pg as indicating FID (Mittman *et al*., 1997), and a CHr < 26 pg assumed to indicate ID as quoted elsewhere (Fishbane *et al*., 1997). This presents an understanding that normal haemoglobinisation means normal iron, but that was not the case in the results obtained for iron index in the normal population in this study. These indicated that, if serum iron were to be used as the key indicator of ID, many of the participants within the normal group would have been classified as having ID.

The diagnostic plots used in this study have several advantages over other diagnostic diagrams. This is because different stages of ID can be identified at quick glance, and a combination of biochemical and haematological parameters can reveal early and progressive ID.

The data in this group represents the population of hospitalised patients with anaemia, rheumatoid arthritis, gastroenteritis, cancer and renal disease at St James's Hospital, Dublin. Because of the mixed diagnoses, and possible existence of various stages of ID, it was difficult to provide stratification for each disease. So as to avoid any sort of bias in the sample selection, analysis

and reporting, the diagnostic plot was used to classify patients based on the results of the traditional markers of iron, that is SF for ID and HB for IDA, which were used to classify patients into two, either as iron deficient or non iron deficient.

Because iron metabolism is a dynamic process and cannot be defined by one or even two biochemical and haematological parameters, it is important to have a complete work-up based on a combination of profiles. Therefore, the assessment of the relationship between the currently used serum SF, haemoglobin versus RBC-He and RBC-He would be important. From this study, the initial assessment shows that RET-He and RBC-He indices could provide improved information over SF, and performed better than HB. SF concentrations were elevated in many of the male and female patients, possibly as a result of inflammation as indicated by CRP > 5mg/L. This suggests that the SF concentration is unreliable in ID with systemic disease.

Thomas *et al*, (2002) used a diagnostic plot, which was referred to as a 'Thomas plot'. The approach has been available to clinicians for the diagnosis of functional ID. This plot provides an excellent aid for differentiating functional ID from the classical ID. The Thomas plot was used in this study to evaluate results for RET-He, RBC-He with HB, if HB (cut-offs female < 11.5 g/dL, and male < 13.5 g/dL) were used to indicate IDA, and when compared with RET-He and RBC-He. There was reasonable agreement as indicated in Figures 3.8, 3.10, 3.28 and 3.29. Kim *et al*. (2008) investigated the accuracy of

the content of haemoglobin in reticulocyte (CHr) in comparison with conventional tests, and the relationship between changes in CHr and HB level after treatment where CHr was found to be a better predictor to the response to anaemia treatment. From the diagnostic plots for both males and females in this study, the quadrants representing patient samples with low HB, and normal RET-He would indicate iron deficiency, but the fact that the RET-He is within the cut-off point defined, suggests that some of the patients have probably commenced treatment for ID. HB from the standard FBC sample represents the HB contents of all the erythrocytes which have a life span of 120 days. This means that it could possibly take at least 120 days for the HB value to return to the reference range. The patient samples within the quadrant represent the advantage of RET-He as a parameter useful in the monitoring of iron treatment.

Some quadrants in the diagnostic plots showed correlation between normal RET-He and normal HB (Figures 3.8, 3.9, 3.26, 3.27, 3.28 and 3.29). The finding shows that these patients are part of the population which are not classed as iron deficient. Also, the quadrants representing patients with low HB and either low RET-He or low RBC-He (Figures 3.8, 3.9 and 3.28), indicates patients are most likely suffering from classic IDA, and are receiving no treatment as yet, hence the reason for the low RET-He. A low RET-He result with normal HB would indicate the very early stages of ID development where the bone marrow is just becoming affected. Although few patients

presented within these quadrants, this combination of result is not impossible and, if recognised, could provide huge advantages for the patient as treatment could be initiated as soon as possible.

To compare RET-He, RBC-He with sTfR, as shown in Figures 3.11, 3.12 and 3.17 in female patients, and Figures 3.23 and 3.25 in male patients, sTfR was particularly unaffected, which is suggestive of the relative insensitivity of sTfR in diagnosing FID. This is in agreement with the study of Briggs *et al*., (2001). Although sTfR has been described as having potential for the iron status evaluation (Punnonen *et al*., 1997), others have rejected the claim and recommend that the interpretation be made carefully, especially with erythroid marrow activity taken into consideration (Siebert *et al*., 2003). sTfR levels can be low in hypo-proliferative states, and is usually the last parameter to react to ID (Siebert *et al*., 2003). Although some patients appear to have a normal sTfR concentration, they may have low iron stores that could go unnoticed, if SF analysis is not performed. Therefore, it would appear that the use of sTfR is limited, and may only be useful in differentiating between ID and ACD (Siebert *et al*., 2003). sTfR can only be recommended as an additional parameter alongside the RET-He and RBC-He in the assessment of iron status.

Tables 3.5 and 3.6 show the sensitivity and specificity of the parameters analysed. As indicated, when SF was used to classify patients as having ID in female patients, a sensitivity of 100.0% and specificity of 99.9% was achieved.

The issue of how much information can be provided by SF and HB depends on many factors, including age, gender and even ethnicity. The use of ZPP level is sensitive in the first line diagnostic test for IDA as during the depletion of iron, the utilisation of zinc increases and the resultant effect is seen in elevated ZPP (Das & Philip, 2008). Although many studies have reported that the ZPP increase compares well with the SF concentration < 20 ng/mL, findings in this study concur with that argument, even though it could potentially become elevated in chronic inflammation, which may limit its diagnostic ability (Hastka, *et al*., 1996).

The ROC curve analysis as shown in Figures 3.5 and 3.6 for female patients, and Figures 3.20 and 3.21 for male patients, showed the AUC_{ROC} for RET-He and RBC-He to be as close as possible to the AUC_{ROC} for SF when it was chosen to classify ID, and also to HB when chosen to classify IDA in both female and male patients.

Using SF as the standard indicator of ID in female patients, the AUC_{ROC} obtained for RET-He was 0.72, sensitivity was 89.6% and specificity was 58.0%. The AUC_{ROC} for RBC-He was 0.72; sensitivity 88.6% and specificity 57.0%. Regardless of the diagnosis, reticulocyte and erythrocyte indices allow haemoglobinisation of RBC changes to be monitored. This information gave an indication of its reliability and usefulness in the early prediction of iron status.

The study also considered MCV in relation to RET-He and RBC-He in the patient population. MCV is the average volume of the entire red cell population, and changes can take a relatively long time. It is recognised to be an insensitive measure of iron deficiency. From the stability study conducted within this project, MCV was indeed found to be unstable and this fact further limits its diagnostic potential. Also, some workers ranked MCH over MCV as a better marker of iron deficiency especially in patients with inflammatory diseases like rheumatoid arthritis. It was also found that many of the patients in this study presented with low MCH in comparison with MCV, which supports the view of O'Broin *et al*., (2005) in their proposal that MCH should be used in conjunction with SF in predicting IDA in patients with inflammatory disorder.

Overall, the data in this study shows RET-He and RBC-He could provide an improved and early diagnosis of ID, providing a FBC sample is drawn in the first place, especially in non anaemic ID, when compared to HB, MCV, HCT, RDW, SF, and sTfR. The acute phase response questions the reliability of biochemical tests such as iron, TSAT and ZPP. SF in particular is a strong positive acute phase reactor and is substantially elevated in inflammatory conditions.

It can be established from this study that the ability of the currently used traditional markers of iron status may be strengthened with the inclusion of RET-He and RBC-He. The correlation between RET-He and RBC-He in all

patient groups was excellent and are both interchangeable for diagnostic purposes.

The understanding gained from this study indicates that RET-He and RBC-He could detect FID in various conditions. It is extremely important to establish a diagnosis in those patients with an inflammatory response, and the fact that RET-He and RBC-He provide the accurate number of individual red cells and reticulocyte analysis using the flow cytometry, is in contrast to MCV, MCH, MCHC which uses the calculation of the mean indices of the red cell, can be very insensitive as shown in this study. The reason is because, by the time the values of the MCV and MCH decline, the ID would have possibly progressed to an advanced stage on most occasions. Also, if this is accompanied by inflammation, measurement of SF and TSAT becomes unreliable. Therefore, the use of RET-He and RBC-He in an algorithm may improve accuracy of diagnosis without the need for additional costly tests. So for basic identification of ID, the author would recommend clinicians to request FBC, with the addition of RET-He and RBC-He in the profile. For complex diagnosis where inflammation is involved, the request for a FBC should include RET-He, RBC-He and ZPP. Separate serum samples should also be sent for sTfR and SF analysis.

The work presented in this thesis identified useful diagnostic information that can be used in the frontline screening, diagnosis and monitoring of ID and FID in a mixed patient population.

Haemochromatosis treatment monitoring was also considered in this study using RET-He and RBC-He in comparison with RBC, HB, HCT, MCV, MCH and MCHC. The diagnosis of haemochromatosis can be made when a patient has an unexplained TSAT > 60% (male) and TSAT >50% (female), and this is supported by the measurement of SF concentration. The differential values obtained for males and females were significant in cases of RBC, HB and HCT. MCV and MCH were found to be sensitive in this study when compared with RBC-He and RET-He. It has also been claimed previously that an elevated MCV and microcytosis in haemochromatosis may be the result of liver disease. It has not been possible to verify this claim in this study because specific complications as a result of haemochromatosis were not considered within this study (Finch & Finch, 1955). However, the results obtained from the treatment monitoring shows all the erythrocytic parameters to be 99% or more sensitive when compared to RET-He and RBC-He in both males and females. Therefore, the possibility that RET-He and RBC-He could be useful in the monitoring of treatment of haemochromatosis patients exists. However, both parameters should be studied in pre and post phlebotomy treatment in the future.

The author wishes to acknowledge the established guidelines for the diagnosis of ID that have been written and implemented by different working groups for clinical practice. The guideline, as provided for by the British

Society of Gastroenterologist in 2007, advised that most of the parameters within the study, such as haemoglobin, SF, and CRP should be used for laboratory screening. It was also recognised that, for patients in remission or with mid disease, at least one measurement of the parameter listed should be carried out every 6-12 months. The guideline also fully recognised that blood loss and chronic diseases could be a result of possible intestinal inflammation. Therefore, CRP and SF were added to the screening profile.

The Inflammatory Bowel Disease working group, formed in 2005, decided on a guideline in 2007 which agreed that the interpretation of the iron indices should be based on modulating factors and should be based on an appropriate cut-off value. The screening parameters as agreed within the framework include the use of HB, SF and CRP. The approach introduced was that a full anaemia work up should commence once the HB level falls below the reference range. The anaemia workup as drawn up includes SF, sTfR, CRP, MCV and MCH. In screening for ID, the guideline provides for a SF level of < 30 ug/L. A distinction was made that if the SF level is between 30 and 100 ug/L, a diagnosis of anaemia of chronic disease (ACD) is likely, even though SF ≤ 100 ug/L is the recommended cut-off. The author of this thesis would like to suggest the addition of RET-He and RBC-He to the guidelines, so as to provide adequate and correct diagnostic information which could be used to monitor therapeutic intervention especially in the category of patients who might have ID or ACD as a result of inflammation.

It was reported in the Ret-He White Paper, written and published by the Sysmex America, that the Kidney Disease Outcomes Quality Initiative recently updated its guidelines, which was driven by the complexities involved in the monitoring of ID associated with chronic kidney disease. The initiative recommended the use of RET-He with the threshold of 29 pg, rather than TSAT.

Although this study did not directly involve clinicians, it does however provide information that would allow them to adequately care for their patients. For example, the Kidney Disease Outcomes Quality Initiative guidelines, published in 2006, caution the administration of iron when SF levels exceed 500 ng/mL. The reason is that the administration of iron could have a potential damaging effect on the treatment outcome. Many other issues relating to long-term safety and cost effectiveness have also been considered. Therefore, to avert the issue of assay reliability and its combination with sensitivity, ease of use and cost effectiveness, the inclusion of RET-He and RBC-He may provide some answers.

It is also important to note that RET-He and RBC-He are not foolproof from limitations, part of it could be the widespread availability, which limit its use, especially in the developing countries and countries where it may be difficult to have the full complement of equipped haematology laboratories.

CHAPTER 5 CONCLUSION AND RECOMMENDATIONS

5.0 Conclusions and implications for professional practice

The findings in this study have explained the use of laboratory methods as an essential aspect of medicine. Although there is a myriad of laboratory tests and procedures available to clinicians, nowadays, it is not every laboratory test offered for a particular diagnostic investigation that will provide a definitive diagnosis.

The work presented in this thesis concludes that the new reticulocyte and erythrocyte parameters RET-He and RBC-He are sensitive predictors of iron deficiency, and can be used to detect changes in cells with inadequate haemoglobinisation. RET-He and RBC-He will have a comfortable place in the screening, diagnosis, treatment and monitoring of iron status. It is suggested as well that the RET-He and RBC-He would be reliable in the evaluation of haemochromatosis patients who are currently undergoing treatment. However, because the study did not consider the complications that may be linked to haemochromatosis, the author wishes to propose the evaluation of RET-He and RBC-He in patients where complications have not occurred. Also,

more work could focus on pre and post iron depletion phlebotomy for comparison with the routinely used erythrocyte indices.

The findings presented here have been implemented in the author's laboratory, and are now available on a routine basis within the Haematology and Nutrition Laboratory of St James's Hospital, Dublin, Ireland.

The contribution of this study to the current awareness is to inform practice and also to indicate that interpretation of various biochemical and haematological indices should be done in the context of the clinical picture, because of the variability in the individual parameter which can make an assay unreliable when screening or diagnosing iron deficiency.

Therefore, the use of RET-He and RBC-He in a diagnostic algorithm would improve accuracy of diagnosis of iron deficiency without the need for additional costly testing. So for basic identification of iron deficiency, the author would recommend clinicians to request FBC, with the addition of RET-He and RBC-He in the profile. For complex diagnosis where inflammation is involved, the request for a full blood count should include RET-He, RBC-He, and ZPP. Separate serum should also be sent for sTfR and SF analysis.

The main question emerging from this study would be to identify the implication of RET-He and RBC-He inclusion into the iron status investigation in practice. Given the fact that there have been several reports on the use of SF, transferrin saturation and sTfR in different diseases groups, it is important

to stress that no single test will exist without its own limitation, and therefore, RET-He and RBC-He are not fool-proof with 100% sensitivity and specificity. However, the findings conclude that both tests are highly sensitive. Also because the so called “gold standard” tests for iron deficiency, that is, SF and others, have serious limitations, the author can conclude that RET-He and RBC-He may serve as the closest parameters in reliably diagnosing ID.

5.1 Change of practice

The practicality and technicality of implementation of the findings in this study has provided some changes within the Haematology and Nutrition Laboratory of the hospital. Given the fact that the St James’s Hospital is the largest teaching hospital in Ireland, the real change tends to require a lot of things ranging from staff, cost and time, because of the overstretched workload. Therefore, the laboratory management would have to weigh the cost versus benefit of any new addition to the current service. Twenty percent of the FBC workload is received from general practice surgeries daily, and the total number received could be in the region of 3000 samples daily, including samples from within the hospital. In order not to add additional cost to the budgetary allocations for the laboratory, a decision was taken to write letters to all the GP surgeries which advised them of the availability of

the tests in the laboratory. This means if a request is needed, tests can be ordered within the test menu on the laboratory request. This will reduce the amount of reagents that would be needed to run the cytometric assays on every sample. The potential to run assays on every sample exists, but to avoid unnecessary cost, the priority is given to the requesters.

5.2 Staff training

Another consideration regarding the implementation of the outcome of this study will be hinged on staff training. The introduction of the RET-He and RBC-He to Sysmex XE2100^{TM} analyser used in the Haematology Laboratory has not been difficult because the assays are available using the technology which is available on the existing analyser. The test is performed using the FBC sample, and in the same way of sample aspiration. The only changes to the operation are the reagent dyes required and the software modification. Therefore, the standard operating procedure will be written, and procedural amendment will be made, which will provide details of changes as part of the on-going training and education for staff.

Also, because assays will be performed usually with a request for a FBC, it is envisaged that there would be no requirement for extra staff at this point in time. Hopefully, the addition of the test will fit well into the existing work

flow and will not have a negative impact on the turn-around time for the request.

5.3 Transfer of the new tests to other laboratories in Ireland

Most Irish laboratories (60%) have either ADVIA 120 or Sysmex XE2100 as the main haematological analyser. These analysers have potentials for measuring CHr, RET-He and RBC-He. ADVIA 120, which was the first haematology analyser used to measure CHr is very popular. Although many of the users do not utilise the feature on the analyser, with the outcome of this study, it is expected that laboratories will provide the test if requested by clinicians.

5.4 Future investigations

In order to examine the area of study further, it would be of interest to follow up requesting clinicians and patients, to study what impact the sole use of RET-He and RBC-He would have on the therapy if they rely on these indices for the iron status assessment.

In order to establish the effective management of iron deficient patients, further work should be undertaken to develop an assessment tool that could be utilised post iron status assessment.

Whilst this study provides quantitative measurement of all the traditional markers of iron deficiency and the new markers, little is known about how clinicians intend to progress the new assays to help in the diagnosis and management, therefore, a qualitative study examining the clinicians pattern of request of iron indices may provide greater understanding of whether there would be a significant impact in the use of these tests.

5.5 Publishing

Throughout this project, the author has undertaken significant learning in relation to iron status and its assessment. This has led to local service improvement and an ongoing expansion of the test repertoire. In addition, the interim findings of this study have been presented at the national level of the Association of Clinical Biochemist in Ireland (ACBI) (Appendix C), and at the annual conference of the Academy of Medical Laboratory Science in Ireland (Appendix B).

The overall findings of this study will produce at least one paper which will be submitted to the Annals of Clinical Biochemistry journal, and also to the British Journal of Biomedical Science. This would allow the findings to have more positive and wider impact on how iron status is assessed.

CHAPTER 6 REFLECTIONS ON PROFESSIONAL DOCTORATE

Fish & Twinn (1997) believe competence in professional practice must embrace skills in new and developing aspects of that professional area. They also argued that today's competence certainly may not be sufficient for tomorrow's discoveries. Based on this and much evidence, learning is more likely to lead to an improvement in professional practice and development.

It has been my ambition to pursue the medical laboratory profession to the highest level of qualification possible. As Eraut (1994) stated, 'qualification is a rite of passage' and a 'landmark in the process of professional socialisation'. Even before I was accepted to study the professional doctorate, I had offers from two universities to study full time PhD by research in completely different aspects of science. Because the two offers were based on a full time study, I thought this would impact on my family and also my ability to continue to work. Therefore, I declined the offers. But because my goal was to focus on the biomedical science area, I became aware of the opportunity to study for a professional doctorate through reading the Biomedical Scientist.

I live and work in the Republic of Ireland. This fact initially delayed the opportunity to meet with the programme manager, Professor Graham Mills. When I finally did in the summer of 2005, he was a little bit concerned about

the logistics involved in coming over to Portsmouth for class attendance, which was required to fulfil the Part 1 of the course. But I was so determined and, rather than visualising and analysing the problem with the logistics of travelling, I saw opportunity. It was indeed difficult, especially having to catch flights late at night and passing nights at a friend's residence in Eastleigh to attend lectures the following morning. But I was glad when the two-weekly flights from Dublin to Southampton were over after two years.

I enjoyed the Part 1 of the programme immensely. On reflection, the taught modules have developed my skills in research methods and analysis. I had opportunity to learn about things that I have never heard about. I found some aspects of the taught programme challenging but enjoyable. I met professionals from different backgrounds, and enjoyed the multidisciplinary interaction with colleagues.

There is a huge difference between conducting research at master's level and doctoral level, because of the complexities involved in designing and conducting research. I was able to build on the modules undertaken at Part I level and, sooner than I thought, with the help of my supervisor Dr Sean O'Broin, it dawned on me that I was on my own and that necessary planning was needed to get things done. I was mentally challenged, when it came to the point of writing the application to the ethics committee to be able to conduct the research. After many letters and meetings, I received an ethics approval to conduct the research, which was the first hurdle crossed to

succeed at this level of study. Overall, the understanding gained at the start of the course really helped me in writing a good proposal, and has enlightened my understanding on how to source funding, select patients, collect and analyse data.

The stages of planning, reading, thinking, observing and setting up racks and ensuring samples get processed before certain times of the day are not fun at all. Especially when I was setting up the sTfR assays using the manual method. Perhaps that is the price you have to pay to undertake the doctoral research study. This is not to scare prospective applicants, but to prepare them for the challenges ahead. On reflection, I naturally gave up on many activities and found solace in staying late and working on the laboratory bench.

Surely, I have always enjoyed working in the laboratory. I have been very fortunate to be able to work at the busiest and largest Hospital in Ireland, and the level of work carried out in the laboratory is rewarding. This afforded me the opportunity to be able to get my research work funded. I also had the opportunity to receive ideas from people with great experience in research and development whose support and encouragement were invaluable.

From a career perspective, I have since had the opportunity to apply for senior and higher positions and received offers. Probably this would not have happened if I had not embarked on the professional doctorate, it is hard to

say. However, the understanding gained has helped me in much of my career decision making.

Overall, apart from the specific scientific objectives which I set out from the time I commenced this study, I have been able to realise the significance of conducting a research to an international standard by applying scientific evidence based research. I have also been able to present part of my work at the national meetings of two major professional bodies in Ireland (AMLS and ACBI). I also hope that there would be no barrier in making new contacts amongst my professional peers which would enable me to explore and disseminate ideas and ultimately become a more advanced practitioner.

As for the future, I hope that I will have the opportunity to disseminate and build on the work described in this thesis. Finally, I look forward to exciting times with attendance at meetings and conferences and, more importantly above work, to spend a wonderful time with my wife and the three children, one of which arrived two years ago when I was still contemplating on how to progress with the project.

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APPENDICES

APPENDIX A: Ethics approval letter

THIS NOTEPAPER MUST NOT BE USED FOR PRESCRIPTIONS OR INVOICING PURPOSES

SJH/AMNCH Research Ethics Committee Secretariat
Dan Lynch Ph: 4142860 email: Dan.Lynch@amnch.ie
Ursula Ryan Ph: 4142342 email: Ursula.Ryan@amnch.ie
Secretariat Fax 4142371

THE ADELAIDE & MEATH HOSPITAL, DUBLIN
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TALLAGHT, DUBLIN 24, IRELAND
TELEPHONE +353 1 4142000

Dr. Sean O'Broin
Department of Haematology
St. James's Hospital
James Street
Dublin 8

June 18th 2008.

REC reference: 2008/05/02 (*Please quote REC reference on all correspondence)*

Re: An Evaluation of a Newly Automated Serum Transferring Receptor Assay and New Erythrocyte Haemoglobinization Indices in the Assessment of Iron Deficiency in a Routine Diagnostic laboratory.

Dear Dr. O'Broin

The SJH / AMNCH Research Ethics Committee, at its meeting on May 28th 2008, agreed to give ethical approval to the above proposed study.

Yours sincerely,

Daniel R. Lynch,
Secretary,
SJH/AMNCH Research Ethics Committee.

APPENDIX B: AMLS Conference 2007 – poster presentation

The Academy of Medical Laboratory Science
31 Old Kilmainham, Dublin 8, Ireland.
Telephone: 01-677 5602
Fax: 01-677 5652
E-Mail: mail@amls.ie
Web Site: www.amls.ie

Mr Aderemi Adelugba
82 Jamestown Park
Ratoath
Co Meath

20th October 2007

CONFERENCE 2007
Sligo Radisson SAS Hotel, Rosses Point, Co. Sligo
9th, 10th & 11th November 2007.
POSTER COMPETITION

Dear **Mr Adelugba,**

Thank you for submitting an abstract of your poster for Conference 2007. The organisation committee is delighted to accept your abstract.

The title I have for your poster is:

AN ASSESSMENT OF RED CELL HAEMOGLOBIN EQUIVALENTS (RET-He and RBC-He) IN SCREENING FOR IRON DEFICIENCY

Please check that this is correct. Please let me know by email if I have any incorrect information.

The board for the poster will be 2 metres high by 1 metre wide.

The venue for the display will be the Benbulben 3/Bar Area in the Radisson Hotel, Sligo. This area can be accessed from 12 noon and the posters need to be in position by 15.30 on Friday, if in competition.

The posters will be displayed from Friday 9th November at 15.30 until Saturday 10th November at 16.00 at which time they must be taken down. **The AMLS will not take responsibility for the posters after 16.00 on Saturday.**
If selected, the presentation on Power Point will consist of five slides maximum, taking no longer than 5 minutes. This will take place between 14.00 and 15.00 on Saturday 10th.

Yours sincerely,

Columba Quinn
AMLS Administrator

APPENDIX C: ACBI Conference 2004 – poster presentation

ACBI 2004 POSTER ABSTRACT FORM

***Title*: Acute Phase Response and Biochemical Assessment of Iron Deficiency.**

***Authors*: Peadar McGing[1], Aderemi Adelugba[2], Edwin Wright[1], Frank Kyne[1].**

***Address*:** Biochemistry Dept., Mater Misericordiae University Hospital, Eccles St., Dublin 7 [1], Biochemistry Dept., Bon Secours Hospital, Glasnevin, Dublin 7. [2].

Introduction: Iron deficiency is commonly screened for using ferritin as the sole biochemical test. Ferritin is preferable to serum iron which is affected by diurnal variation and food intake. However as a measure of iron stores, ferritin may itself be unreliable in inflammatory conditions, it being an acute phase protein. We undertook a study to investigate the accuracy of ferritin as a measure of iron deficiency in the presence of an acute phase response.

Materials and Methods: 65 patients attending the Bon Secours Hospital were tested for iron deficiency using biochemical (ferritin, iron, TIBC, iron saturation) and haematological (Hb, MCV, RDW) parameters. C-Reactive Protein (CRP) was the parameter chosen to indicate acute phase response in patients. Patients were selected to include individuals with different disease states likely to have or not to have an acute phase response.

Results: Of the 65 individuals tested for query-iron-deficiency, 53 (82%) were deemed to have low iron status. All 53 patients had iron saturation below 22% and serum iron below 9.5µmol/L, but only 33 of these (62%) had ferritin levels below the reference range (14ug/L; Abbott Axsym). 20 patients in whom iron deficiency was strongly indicated by other diagnostic criteria had normal or raised ferritin levels. Of these 20 patients, 14 (70%) had elevated CRP levels (>5mg/L; Roche Integra 800). Three patients whose ferritin levels were raised above the upper reference limit (233µg/L) all had elevated CRP.

Discussion: These results support the proposal that ferritin's behaviour as an acute phase protein may make it unreliable in excluding iron deficiency in individuals with inflammation / acute phase response.

DEADLINE FOR SUBMISSION: Friday, 25th June 2004

APPENDIX D: Roche Modular insert for iron analysis

1965239001V8

Fe

Iron

Roche

● Indicates Roche/Hitachi analyzer(s) on which kit(s) can be used

Cat. No.	Bottle	Contents	717	747	747-400	902	904	911 912	917	MOD P	MOD D
11876996	1	REAGENT 6 x 64 mL							●	●	
	2	REAGENT 6 x 16 mL									
11929658	1	REAGENT 6 x 258 mL								●	●
11929666	2	REAGENT 6 x 68 mL									
11929674	1	REAGENT 4 x 641 mL									●
11929682	2	REAGENT 4 x 278 mL									
11970704	1	REAGENT 12 x 50 mL	●			●	●	●			
	2	REAGENT 6 x 20 mL									
11970747	1	REAGENT 6 x 100 mL	●				●	●			
	2	REAGENT 3 x 46 mL									
11970771	1	REAGENT 5 x 500 mL		●							
11970798	2	REAGENT 5 x 200 mL									
11970828	1	REAGENT 4 x 1020 mL			●						
11970844	2	REAGENT 4 x 430 mL									

Some analyzers and kits shown may not be available in all countries. For additional system applications, contact your local Roche Diagnostics representative.

English

System information
For Roche/Hitachi 904/911/912/917/MOD P/MOD D: ACN 661

Intended use
In vitro assay for the quantitative determination of iron in human serum and plasma on Roche automated clinical chemistry analyzers.

Summary[1-5]
Ingested iron is mainly absorbed in the form of Fe^{2+} in the duodenum and upper jejunum. The trivalent form and the heme-bound Fe^{2+} component of iron in food has to be reduced by vitamin C. About 1 mg of iron is assimilated daily. Upon reaching the mucosal cells, Fe^{2+} ions become bound to transport substances. Before passing into the plasma, these are oxidized by ceruloplasmin to Fe^{3+} and bound to transferrin in this form. The transport of Fe ions in blood plasma takes place via transferrin-iron complexes. A maximum of 2 Fe^{3+} ions per protein molecule can be transported. Serum iron is almost completely bound to transferrin.
Iron (non-heme) measurements are used in the diagnosis and treatment of diseases such as iron deficiency anemia, hemochromatosis (a disease associated with widespread deposit in the tissue of the two iron-containing pigments, hemosiderin and hemofuscin, and characterized by pigmentation of the skin), and chronic renal disease. Iron determinations are performed for the diagnosis and monitoring of microcytic anemia (e.g. due to iron metabolism disorders and hemoglobinopathy), macrocytic anemia (e.g. due to vitamin B12-deficiency, folic acid deficiency and drug-induced metabolic disorders of unknown origin) as well as normocytic anemias such as renal anemia (erythropoetin deficiency), hemolytic anemia, hemoglobinopathy, bone marrow disease and toxic bone marrow damage.
Numerous photometric methods have been described for the determination of iron. All have the following in common:

- Liberation of Fe^{3+} ions from the transferrin complex using acids or detergents.
- Reduction of Fe^{3+} ions to Fe^{2+} ions.
- Reaction of the Fe^{2+} ions to give a colored complex.

The method described here is based on the FerroZine method without deproteinization.

Test principle SPECTROPHOTOMETRIC
Colorimetric assay

- Sample and addition of R1 (buffer/detergent)
- Addition of R2 (ascorbate/FerroZine) and start of reaction:

$$\text{Transferrin-Fe-complex} \xrightarrow{\text{pH} < 2.0} \text{apotransferrin} + Fe^{3+}$$

$$Fe^{3+} \xrightarrow{\text{ascorbate}} Fe^{2+}$$

$$\text{FerroZine} + Fe^{2+} \longrightarrow \text{colored complex}$$

Under acidic conditions, iron is liberated from transferrin. Lipemic samples are clarified by the detergent. Ascorbate reduces the released Fe^{3+} ions to Fe^{2+} ions which then react with FerroZine to form a colored complex. The color intensity is directly proportional to the iron concentration and can be measured photometrically.

Reagents – working solutions
R1 Citric acid: 200 mmol/L; thiourea: 115 mmol/L; detergent.
R2 Sodium ascorbate: 150 mmol/L; FerroZine: 6 mmol/L; preservative.

Precautions and warnings
For in vitro diagnostic use.
Exercise the normal precautions required for handling all laboratory reagents.
Warning: This reagent contains thiourea, a substance known to the State of California to cause cancer or reproductive harm. It may also cause skin reactions. In the event of contact, flush affected areas with copious amounts of running water. Get immediate medical attention for contact with the eyes or if ingested.
Safety data sheet available for professional user on request.
Disposal of all waste material should be in accordance with local guidelines.

Reagent handling
R1: Ready for use
R2: Ready for use

Storage and stability
Unopened kit components: Up to the expiration date at 2-8°C
R1: 28 days opened and refrigerated on the analyzer
R2: 28 days opened and refrigerated on the analyzer. Store protected from light.

Specimen collection and preparation
Only the specimens listed below were tested and found acceptable.
Serum: Collect serum using standard sampling tubes.
Plasma: Li-/Na-/NH_4^+-heparin plasma. EDTA and oxalate plasma cause decreased values.
When processing samples in primary tubes, follow the instructions of the tube manufacturer.

Stability[6]: 7 days at 15-25°C
3 weeks at 2-8°C
several years at(-15) - (-25)°C

Separate serum or plasma from the clot or cells within 1 hour.
Centrifuge samples containing precipitates before performing the assay.

Materials provided
See "Reagents – working solutions" section for reagents.

October 2010

Fe

Iron

cobas®

● Indicates Roche/Hitachi analyzer(s) on which kit(s) can be used

Cat. No.	Bottle	Contents	902	904	911 912	917	MODULAR P	MODULAR D
11876996 216	1	REAGENT 6 x 64 mL				●	●	
	2	REAGENT 6 x 16 mL						
11929658 216	1	REAGENT 6 x 258 mL					●	●
11929666 216	2	REAGENT 6 x 68 mL						
11970704 216	1	REAGENT 12 x 50 mL	●	●	●			
	2	REAGENT 6 x 20 mL						
11970747 216 (US only)	1	REAGENT 6 x 100 mL			●			
	2	REAGENT 3 x 46 mL						

Some analyzers and kits shown may not be available in all countries. For additional system applications, contact your local Roche Diagnostics representative.

English

System information
For Roche/Hitachi 904/911/912/917/MODULAR P/MODULAR D analyzers: ACN 661

Intended use
In vitro assay for the quantitative determination of iron in human serum and plasma on Roche automated clinical chemistry analyzers.

Summary[1,2,3,4,5]
Ingested iron is mainly absorbed in the form of Fe^{2+} in the duodenum and upper jejunum. The trivalent form and the heme-bound Fe^{3+} component of iron in food has to be reduced by vitamin C. About 1 mg of iron is assimilated daily. Upon reaching the mucosal cells, Fe^{2+} ions become bound to transport substances. Before passing into the plasma, these are oxidized by ceruloplasmin to Fe^{3+} and bound to transferrin in this form. The transport of Fe ions in blood plasma takes place via transferrin-iron complexes. A maximum of 2 Fe^{3+} ions per protein molecule can be transported. Serum iron is almost completely bound to transferrin.
Iron (non-heme) measurements are used in the diagnosis and treatment of diseases such as iron deficiency anemia, hemochromatosis (a disease associated with widespread deposit in the tissue of the two iron-containing pigments, hemosiderin and hemofuscin, and characterized by pigmentation of the skin), and chronic renal disease. Iron determinations are performed for the diagnosis and monitoring of microcytic anemia (e.g. due to iron metabolism disorders and hemoglobinopathy), macrocytic anemia (e.g. due to vitamin B12-deficiency, folic acid deficiency and drug-induced metabolic disorders of unknown origin) as well as normocytic anemias such as renal anemia (erythropoetin deficiency), hemolytic anemia, hemoglobinopathy, bone marrow disease and toxic bone marrow damage.
Numerous photometric methods have been described for the determination of iron. All have the following in common:

- Liberation of Fe^{3+} ions from the transferrin complex using acids or detergents.
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- Reaction of the Fe^{2+} ions to give a colored complex.

The method described here is based on the FerroZine method without deproteinization.

Test principle
Colorimetric assay

- Sample and addition of R1 (buffer/detergent)
- Addition of R2 (ascorbate/FerroZine) and start of reaction:

$$\text{Transferrin-Fe-complex} \xrightarrow{\text{pH} < 2.0} \text{apotransferrin} + Fe^{3+}$$

$$Fe^{3+} \xrightarrow{\text{ascorbate}} Fe^{2+}$$

$$\text{FerroZine} + Fe^{2+} \longrightarrow \text{colored complex}$$

Under acidic conditions, iron is liberated from transferrin. Lipemic samples are clarified by the detergent. Ascorbate reduces the released Fe^{3+} ions to Fe^{2+} ions which then react with FerroZine to form a colored complex. The color intensity is directly proportional to the iron concentration and can be measured photometrically.

Reagents – working solutions
R1 Citric acid: 200 mmol/L; thiourea: 115 mmol/L; detergent.
R2 Sodium ascorbate: 150 mmol/L; FerroZine: 6 mmol/L; preservative.

Precautions and warnings
For in vitro diagnostic use.
Exercise the normal precautions required for handling all laboratory reagents.
Safety data sheet available for professional user on request.
Disposal of all waste material should be in accordance with local guidelines.
Required for US:
Warning: This reagent contains thiourea, a substance known to the State of California to cause cancer or reproductive harm. It may also cause skin reactions. In the event of contact, flush affected areas with copious amounts of running water. Get immediate medical attention for contact with the eyes or if ingested.

Reagent handling
R1: Ready for use
R2: Ready for use

Storage and stability
Unopened kit components: Up to the expiration date at 2–8 °C
R1: 28 days opened and refrigerated on the analyzer
R2: 28 days opened and refrigerated on the analyzer. Store protected from light.

Specimen collection and preparation
For specimen collection and preparation only use suitable tubes or collection containers.
Only the specimens listed below were tested and found acceptable.
Serum
Plasma: Li-/Na-/NH_4+-heparin plasma.
EDTA and oxalate plasma cause decreased values.

The sample types listed were tested with a selection of sample collection tubes that were commercially available at the time of testing, i.e. not all available tubes of all manufacturers were tested. Sample collection systems from various manufacturers may contain differing materials which could affect the test results in some cases. When processing samples in primary tubes (sample collection systems), follow the instructions of the tube manufacturer.

Stability:[6] 7 days at 15–25 °C
3 weeks at 2–8 °C
several years at (-15)–(-25) °C

Separate serum or plasma from the clot or cells within 1 hour.
Centrifuge samples containing precipitates before performing the assay.

Materials provided
See "Reagents - working solutions" section for reagents.

Materials required (but not provided)

- Calibrator: C.f.a.s. (Calibrator for automated systems), Cat. No. 10759350 190, 10759350 360 (for USA):
- Controls: Precinorm U, e.g. Cat. No. 10171743 122, or Precinorm U plus, Cat. No. 12149435 122, 12149435 160 (for USA); Precipath U, e.g. Cat. No. 10171778 122, or Precipath U plus, Cat. No. 12149443 122, 12149443 160 (for USA)
- 0.9 % NaCl

APPENDIX E: SYSMEX XE2100 hard copy showing FBC, RET-He & RBC-He

APPENDIX F: Stability data HGB, MCV, MCH, MCHC, RET-Y and RBC-Y

Sample	Day	Temp °C	HGB (g/dL)	MCV(fL)	MCH(Pg)	MCHC(g/dL)	RET-Y(ch)	RBC-Y(ch)
S1	1	RT	6.8	69	20.6	29.7	124.6	115.2
S1	2	4°C	7.7	67.3	20.4	31.2	124.4	115.6
S1	3	20°C	8	68.6	20.9	30.5	126	115.3
S1	4	30°C	7.6	76	21.3	28.3	126.9	116.1
S2	1	RT	14.9	94.7	31.5	33.3	189.1	176.3
S2	2	4°C	15.3	94.6	31.8	33.5	189.3	177.7
S2	3	20°C	15.3	97.7	31.8	32.8	189.1	178.6
S2	4	30°C	15.5	104.8	32	31.1	189.5	178
S3	1	RT	13.2	97.7	33.6	34.4	189.6	178.1
S3	2	4°C	13.4	95	33.5	35.4	189.1	178.4
S3	3	20°C	13.4	98.8	33.7	33.3	188	177.9
S3	4	30°C	13.3	108.4	34.7	30.4	182	177.7
S4	1	RT	12	77.2	25.1	32.4	169.4	157.8
S4	2	4°C	12.4	76.2	25.3	32.9	171.1	157.3
S4	3	20°C	12.4	79	25.4	31.5	170.6	158.1
S4	4	30°C	12.3	86.5	26.5	28.7	170.9	158.3
S5	1	RT	11.1	74.5	23	30.9	161.4	149.6
S5	2	4°C	11.5	73.7	22.8	31.5	161.7	149.3
S5	3	20°C	11.4	77.1	23.3	23.3	162.1	150.1
S5	4	30°C	11.4	80.2	23	28.6	161.4	150.3
S6	1	RT	15.4	78.1	26.7	34.2	180.4	173.3
S6	2	4°C	15.6	78.3	26.8	34.1	180.6	173.3
S6	3	20°C	15.7	82.8	26.7	32.3	180.9	173.2

S6	4	30°C	15.7	87.2	27.1	30.8	180.3	273.7
S7	1	RT	12.3	98.1	33.1	33.7	171.8	156
S7	2	4°C	13.2	96.7	33.4	34.6	171.6	156.1
S7	3	20°C	12.9	100.3	32.9	32.8	171	156.6
S7	4	30°C	12.5	107.1	32.5	34.4	171.1	157
S8	1	RT	13.9	96.1	31.8	33.1	190.5	177
S8	2	4°C	14.5	95.6	31.8	33.3	190.3	177.3
S8	3	20°C	14.3	99.6	31.6	31.8	190.5	177.1
S8	4	30°C	14.5	105.7	32	30.3	190.8	177.4
S9	1	RT	11.1	96.7	32	33.1	180	164.4
S9	2	4°C	11.3	97.4	32.3	33.2	181.3	165.3
S9	3	20°C	11.4	99.1	32.4	33.6	180.6	165.1
S9	4	30°C	11.3	104.5	32.2	32.7	180.8	164.8
S10	1	RT	8.6	87.3	27.2	31.2	166.9	160.1
S10	2	4°C	8.8	88.5	27.6	31.4	166.1	159.3
S10	3	20°C	8.8	96.7	27.5	31.6	167.2	160.2
S10	4	30°C	9	99.7	27.9	31.8	167.3	161.4
S11	1	RT	11.3	85.7	23.3	27.3	188.1	175.6
S11	2	4°C	11.5	88.1	23.6	28	187.4	175.8
S11	3	20°C	11.7	93.6	23.7	27.6	188.1	174.6
S11	4	30°C	11.3	97.4	23.8	27.5	188.8	175.1
S12	1	RT	9.6	100	29.2	28.7	178.3	168.1
S12	2	4°C	9.7	102.3	29.5	28.5	178.4	168.3
S12	3	20°C	9.4	105.4	29.1	28.4	177.4	168.7
S12	4	30°C	9.8	108.4	29	28.1	177.6	168.4
S13	1	RT	10.4	92.7	29.1	31.5	165.3	155.6

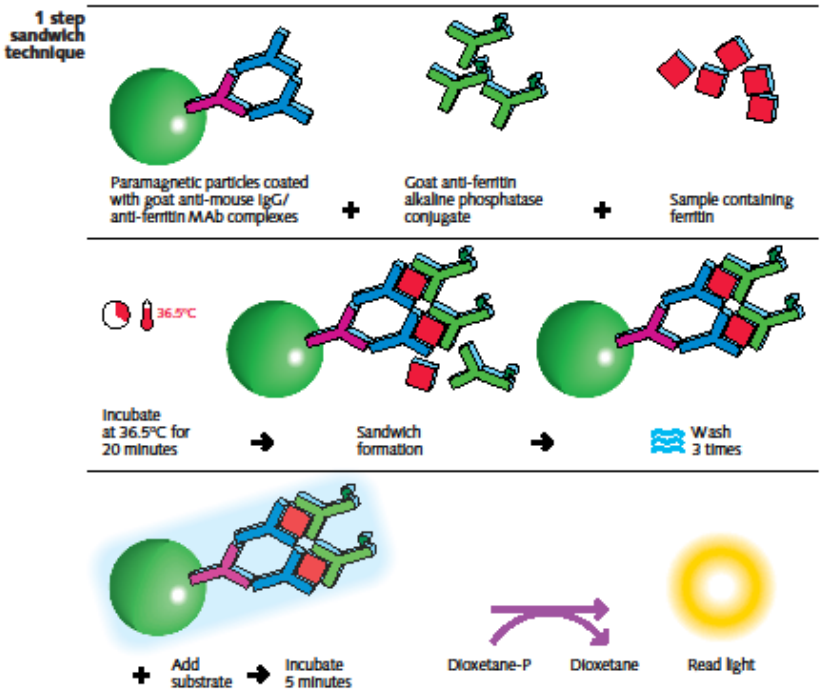
S13	2	4°C	10.4	95.1	29.3	31.7	166	155.4
S13	3	20°C	10.7	98.1	29.1	31.3	165.4	156.7
S13	4	30°C	10.3	99.3	29.6	31.5	165.7	155.3
S14	1	RT	7.9	117.6	20.7	17.6	180.7	172.3
S14	2	4°C	7.7	118.6	20.6	17.8	181.1	173
S14	3	20°C	7.7	119	20.8	17.5	180.4	172.6
S14	4	30°C	7.5	119.2	21.2	17.9	180	173.1
S15	1	RT	14.5	91.6	29.7	32.4	178.5	165.4
S15	2	4°C	14.3	93.4	29.5	32.6	178.1	164.8
S15	3	20°C	14.4	95.9	29.7	32.8	179.1	164.9
S15	4	30°C	14.1	97.1	29.5	32.5	178.8	166

APPENDIX G: **Beckmann Coulter Access principles of serum ferritin assay**

Bulletin 9075d

Ferritin

- *Excellent sensitivity: 0.2 ng/mL*
  - iron-deficiency anemia
- *Broad dynamic range: 0.2 - 1500 ng/mL*
- *Precision: < 10% CV*
- *28-day open-pack and calibration stability*
- *Calibration stability: 28 days*
- *Proven lot-to-lot consistency*



Signal produced is directly proportional to the ferritin concentration in the sample

APPENDIX H: R&D Systems Quantikine® IVD analysis for sTfR, including plates and graphs.

PRINCIPLE OF THE ASSAY

This assay is based on the microplate sandwich enzyme immunoassay technique using two different monoclonal antibodies specific for sTfR. Samples or standards are pipetted into wells of a microplate pre-coated with a monoclonal antibody that can capture sTfR, thereby immobilizing sTfR to the well. After washing away any unbound protein, a second anti-sTfR monoclonal antibody conjugated to horseradish peroxidase is added. The conjugated antibody completes the sandwich. After washing away unbound conjugated antibody, the amount of conjugate remaining in the well is proportional to the amount of sTfR initially captured. The amount of conjugated enzyme in the well is measured by incubation with a chromogenic substrate.

LIMITATIONS OF THE PROCEDURE

- FOR *IN VITRO* DIAGNOSTIC USE.
- No drugs have been investigated for assay interference.
- The kit should not be used beyond the expiration date on the kit label.
- If samples generate values higher than the highest standard, dilute the samples with the Specimen Diluent and repeat the assay or report the values as > 80 nmol/L.
- Any variation in Specimen Diluent, operator, pipetting technique, washing technique, incubation time or temperature, or kit age can cause variation in binding.

REAGENTS

SORB **sTfR Microplate** (Part 890429) - 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against sTfR.

CONJ **sTfR Conjugate** (Part 890430) - 11.5 mL of mouse monoclonal antibody against sTfR conjugated to horseradish peroxidase, containing red dye and preservative.

CAL **sTfR Standard Set** (Part 890312-890317) - 6 vials of human sTfR in 0.2 mL buffered animal serum with preservative. The concentration of sTfR is shown on the label.

CON **sTfR Control Set** (Part 895426-895428) - 3 vials of lyophilized human sTfR in buffered animal serum with preservative.

DIL SPE **sTfR Specimen Diluent** (Part 895429) - 5 mL of buffered animal serum with preservative.

DIL AS **sTfR Assay Diluent** (Part 895430) - 11 mL of a buffered protein base, containing blue dye and preservative.

BUF WASH 25X **Wash Buffer Concentrate** (Part 895199) - 21 mL of a 25-fold concentrated solution of buffered surfactant with preservative.

SUBS **Substrate** (Part 895431) - 12 mL of stabilized substrate solution.

SOLN STOP **Stop Solution** (Part 895432) - 11 mL of 1 N HCl.

Plate Covers - 4 adhesive plate sealers.

Data Card - Provides ranges of controls.

STORAGE

Unopened Kit	Store at 2 - 8° C. Do not use past kit expiration date.	
Opened/ Reconstituted Reagents	Diluted Wash Buffer	May be stored for up to 1 month at 2 - 8° C.*
	Stop Solution	
	sTfR Specimen Diluent	
	sTfR Assay Diluent	
	sTfR Conjugate	
	Substrate	
	Standards	
	Controls	
	Microplate Wells	Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 2 - 8° C.*

*Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Multi-channel pipette, squirt bottle, manifold dispenser, or automated microplate washer.
- 500 mL graduated cylinder for preparation of Wash Buffer.
- Vortex mixer.

ASSAY PROCEDURE SUMMARY

1. Prepare all reagents as instructed.
2. Add 100 µL sTfR Assay Diluent to each well.
3. Add 20 µL Standard, sample, or Control to each well. **Ensure sample addition is uninterrupted and completed within 15 minutes.** Incubate 1 hr. RT (18 - 25° C)
4. Aspirate and wash 4 times.
5. Add 100 µL Conjugate to each well. Incubate 1 hr. RT
6. Aspirate and wash 4 times.
7. Add 100 µL Substrate to each well. Incubate 30 min. RT
8. Add 100 µL Stop Solution to each well. Read at 450 nm within 30 min. λ correction 540 or 570 nm

CALCULATION OF RESULTS

Average the duplicate readings for each standard, control and sample and subtract the average zero standard optical density.

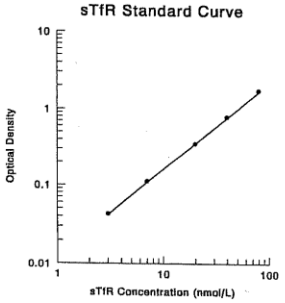
Plot the optical density for the standards versus the concentration of the standards and draw the best curve. The data can be linearized by using log/log paper, and regression analysis may be applied to the log transformation.

To determine the sTfR concentration of each sample, first find the absorbance value on the y-axis and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the x-axis and read the corresponding concentration. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

For values above the highest standard (80 nmol/L), the sample can be reported as >80 nmol/L, or it can be diluted with Specimen Diluent and re-analyzed. Values below the lowest standard (3 nmol/L) should be reported as < 3 nmol/L. Values should not be extrapolated outside the range of the standard curve.

TYPICAL DATA

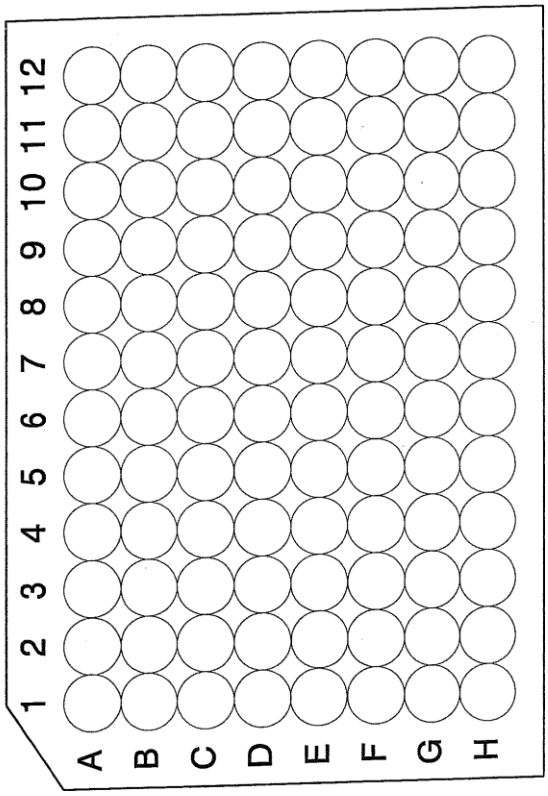
This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



nmol/L	O.D.	Mean	Corrected
0	0.012		
	0.013	0.012	—
3	0.055		
	0.056	0.056	0.044
7	0.123		
	0.125	0.124	0.112
20	0.365		
	0.367	0.366	0.354
40	0.825		
	0.793	0.809	0.797
80	1.756		
	1.777	1.766	1.754

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



14

CONTROL SERUM/SERUM TEMOIN/KONTROLLSERUM

20/2/07

Analyte/Analyte/Faktor: sTfR

Control/Témoin/Kontrolle Lot No./No. de Lot/Lot Nr Expiry Date/Date de Péremption/Verfallsdatum Range/Gamme/Bereich	**1**	236427 04 Oct 07 5.1-7.8 nmol/L
Control/Témoin/Kontrolle Lot No./No. de Lot/Lot Nr Expiry Date/Date de Péremption/Verfallsdatum Range/Gamme/Bereich	**2**	236427 04 Oct 07 13.2-20.1 nmol/L
Control/Témoin/Kontrolle Lot No./No. de Lot/Lot Nr Expiry Date/Date de Péremption/Verfallsdatum Range/Gamme/Bereich	**3**	236427 04 Oct 07 32.8-47.7 nmol/L

Store at 2-8° C/Conserver à 2-8° C/Lagerung bei 2-8° C

See package insert for reconstitution instructions
Voir le protocole d'instructions pour la reconstitution des Témoins
Anweisungen des Rekonstitutions-Protokolls beachten

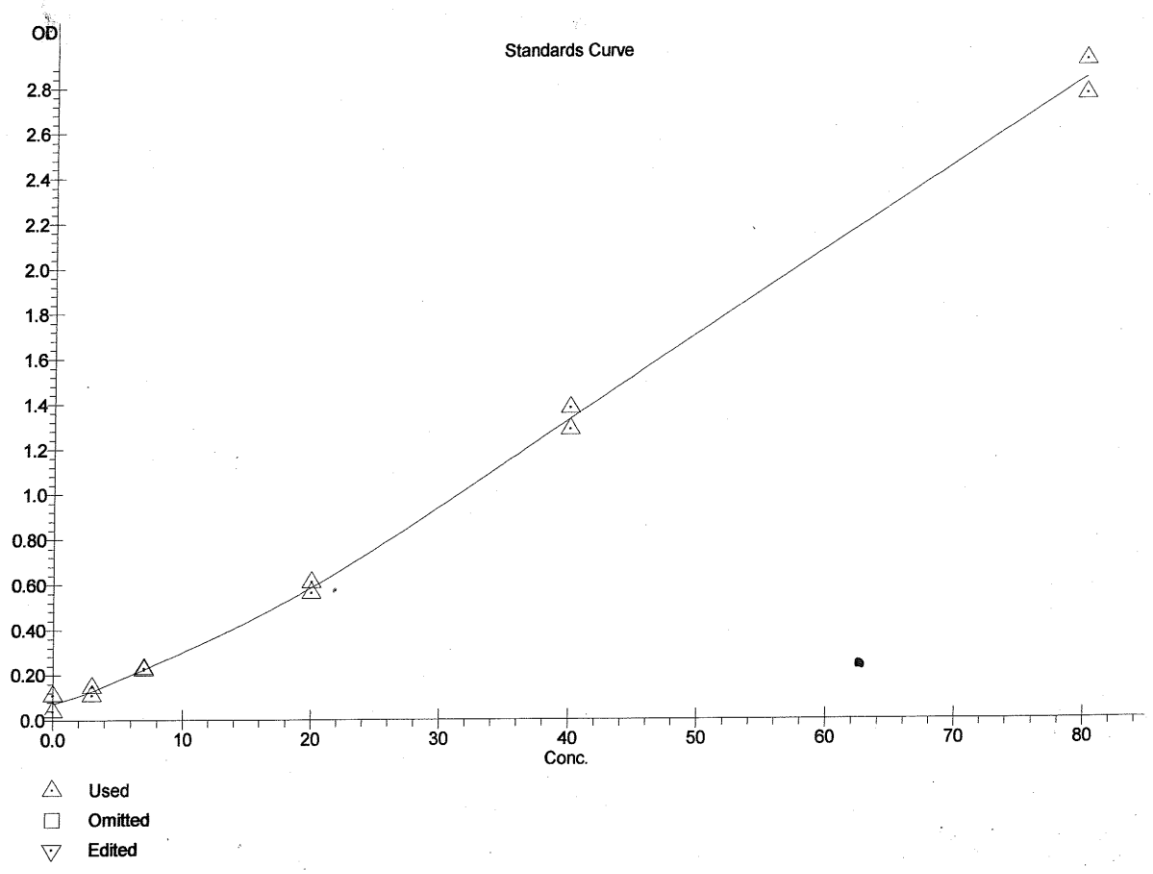
Rev. 750228.3

R&D SYSTEMS

LABSYSTEMS GENESIS V2.16 Results Monday, 27 November 2006 17:16
Raw data filename : c:\genesis\protocol\txrec\27nov06w.001
Processed by Protocol : c:\genesis\protocol\txrec.prt
Plate layout file : c:\genesis\protocol\txrec.plt
Reading type : Dual Wavelength
Instrument version : Multiskan RC V1.5-0
Filter 1 : 450nm
Filter 2 : 570nm
Lag time : 00:00:00
Interval between rdgs : 00:00:10
Mix : NO

Raw data values (calculated):

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.110	1.380	0.440	0.653	0.488	0.671	0.609	1.096	0.657	0.701	0.318	0.468
B	0.039	1.285	0.364	0.659	1.145	0.685	0.391	0.713	1.351	0.511	0.482	0.938
C	0.147	2.764	0.612	0.498	0.238	0.365	0.492	0.532	0.766	0.337	0.566	0.517
D	0.111	2.916	0.371	0.460	0.667	0.317	1.056	0.529	0.340	0.482	0.908	0.311
E	0.223	0.165	0.338	0.504	0.446	0.446	0.797	0.550	0.439	0.360	0.574	1.090
F	0.228	0.145	0.540	0.873	0.661	0.517	0.461	0.842	0.677	0.487	0.316	0.644
G	0.611	0.412	0.465	0.597	0.606	0.453	0.519	0.690	0.500	0.303	0.437	1.249
H	0.563	0.498	0.544	0.504	0.643	1.044	0.927	0.531	2.053	0.756	0.451	1.288



QLog(z) = InverseSinh(z) = Log(z + sqrt(z^2 + 1))
Log(z) is the natural logarithm.
Simple cubic spline (exact fit)
Weighted : NO; summed (residuals)^2 = 0.000000

Standards curve used:

STANDARD		Status	Absorb.	Conc.	Unit	%CV	Std Dev
0.000	A01		0.110	2.090			
	B01		0.039	0.000			
Mean			0.075	1.045	Conc.	141.42	1.478

3.000	C01	0.147	3.790			
	D01	0.111	2.140			
Mean		0.129	2.965	Conc.	39.347	1.167
7.000	E01	0.223	6.898			
	F01	0.228	7.102			
Mean		0.226	7.000	Conc.	2.052	0.144
20.000	G01	0.611	20.728			
	H01	0.563	19.255			
Mean		0.587	19.992	Conc.	5.208	1.041
40.000	A02	1.380	41.224			
	B02	1.285	38.788			
Mean		1.332	40.006	Conc.	4.305	1.722
80.000	C02	2.764	77.995			
	D02	2.916	82.028			
Mean		2.840	80.011	Conc.	3.564	2.851

UNKNOWNS

U1 :

Well	Absorb.	Conc.	Unit	%CV	Std Dev
E02	0.165	4.545			
F02	0.145	3.704			
Mean	0.155	4.129	Conc.	14.403	0.595

Control 1

U2 :

Well	Absorb.	Conc.	Unit	%CV	Std Dev
G02	0.412	14.188			
H02	0.498	17.152			
Mean	0.455	15.696	Conc.	13.359	2.097

Control 2

U3 :

Well	Absorb.	Conc.	Unit	%CV	Std Dev
A03	0.440	15.176			
B03	0.364	12.429			
Mean	0.402	13.830	Conc.	14.051	1.943

U4 :

Well	Absorb.	Conc.	Unit	%CV	Std Dev
C03	0.612	20.763			
D03	0.371	12.692			
Mean	0.491	16.935	Conc.	33.744	5.715

U5 :

Well	Absorb.	Conc.	Unit	%CV	Std Dev
E03	0.338	11.446			
F03	0.540	18.524			
Mean	0.439	15.145	Conc.	33.082	5.010

U6 :

Well	Absorb.	Conc.	Unit	%CV	Std Dev
G03	0.465	16.044			
H03	0.544	18.657			
Mean	0.504	17.372	Conc.	10.636	1.848

U7 :

Well	Absorb.	Conc.	Unit	%CV	Std Dev
A04	0.653	21.983			
B04	0.659	22.162			
Mean	0.656	22.072	Conc.	0.575	0.127

U8 :

Well	Absorb.	Conc.	Unit	%CV	Std Dev
C04	0.498	17.152			
D04	0.460	15.873			
Mean	0.479	16.517	Conc.	5.476	0.905

U9 :

Well	Absorb.	Conc.	Unit	%CV	Std Dev
E04	0.504	17.355			
F04	0.873	28.089			
Mean	0.689	23.003	Conc.	33.042	7.601

U10 :

Well	Absorb.	Conc.	Unit	%CV	Std Dev
G04	0.597	20.304			
H04	0.504	17.355			
Mean	0.551	18.857	Conc.	11.061	2.086

APPENDIX I: Pilot study results - (RET-He and sTfR in ACD and ID)

Variables	**Male (n= 55)** **Mean difference (SD)**	**Female (n = 82)** **Mean difference (SD)**
Haematological parameters	6 (23)	6.1 (28)
Serum ferritin	104 (24.3)	67 (26.8)